LIBERATION OF AMINO ACIDS FROM RAW AND HEATED CASEIN BY ACID AND ENZYME HYDROLYSIS*

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Earlier work showed that the nutritive value of casein was decreased by dry heat and that the addition of lysine restored its nutritive value (1). The lysine was not damaged materially by the heat treatment, since analyses of acid hydrolysates of heated casein with lysine decarboxylase (2) and chemical isolation as the picrate (3) showed no decrease in the lysine content. Eldred and Rodney (2), using the lysine decarboxylase method, and Pader, Melnick, and Oser (4), using the *Streptococcus faecalis* assay, found that heating casein in a dry state at 150° for a few hours decreased the quantity of lysine liberated by enzyme hydrolysis *in vitro*. Block, Jones, and Gersdorff (3) reported that the lysine content of casein was not affected by exposure to dry heat at a temperature at 150°, but that enzymatic liberation of the amino acid was decreased.

Melnick, Oser, and Weiss (5) pointed out that factors known to increase the nutritive value of soy bean protein also increase its *in vitro* digestibility. In a recent report, Riesen *et al.* (6) showed that the degree of liberation of the ten essential amino acids from soy bean oil meal by pancreatin was increased when the meal had been autoclaved for 4 minutes at 15 pounds pressure. When the period of autoclaving was extended to 4 hours, the liberation of these amino acids was decreased below that obtained with the raw meal. The amino acid content was unaffected by the short autoclaving procedure; after prolonged heat treatment, the lysine, arginine, and tryptophan values found by microbiological assay of acid or alkaline hydrolysates were decreased.

To determine whether casein was altered similarly, the effect of heat treatment on the amino acid composition and the extent of liberation of amino acids by enzymes were measured microbiologically. Since preliminary experiments indicated that this protein was much more resistant to changes in digestibility by moist heat than soy bean protein, the longer period of autoclaving was extended from 4 to 20 hours. In this work, the

release of amino acids by successive treatment with a number of mammalian digestive enzymes was investigated.

EXPERIMENTAL

Preparation of Samples—Pyrex trays were filled to a depth of 0.5 inch with vitamin test casein (Smaco) and heated in an autoclave at 15 pounds pressure (121°) for 4 minutes and for 20 hours. The heated casein was then dried in a stream of air at 65° for 24 hours. Moisture and Kjeldahl nitrogen determinations were made on each sample.

Acid and Alkaline Hydrolysis—The conditions of acid hydrolysis which released maximum quantities of amino acids from casein, as measured by the formol titration, were determined in preliminary experiments. Casein was autoclaved with 25 volumes of 3 to 5 N hydrochloric acid at 15 pounds pressure for $1\frac{1}{2}$ to 18 hours; maximum liberation of amino groups was obtained with 3 N acid for 10 hours. Since longer periods of hydrolysis caused a slight reduction of the formol titration value, the 10 hour period with 3 N HCl was adopted for the assay of all amino acids except cystine, tryptophan, and tyrosine.

Riesen (7) found that free cystine was destroyed by the acid hydrolysis procedure used for the release of other amino acids and that maximum cystine values were obtained when the casein was autoclaved with 30 volumes of 2 N hydrochloric acid for 3 hours. This procedure, used in these studies for the hydrolysis for cystine analysis, should give comparable values for the three casein samples, though they may be somewhat lower than the true cystine content.

Alkaline hydrolysates for tryptophan and tyrosine assays were prepared by autoclaving samples of casein with 20 volumes of 5 N sodium hydroxide for 15 hours at 15 pounds pressure. Complete racemization was assumed.

Enzyme Hydrolysis—In enzyme digestion studies, commercial preparations of pancreatic and ereptic enzymes from several sources were assayed for their relative proteolytic or peptidase activities by measuring with formol titration the amino groups liberated from unheated casein. For proteinase activity determinations, 1 gm. of casein was shaken at 37° for 4 hours with 20 mg. of the preparation to be tested at pH 8 with 50 ml. of carbonate buffer. The substrate for peptidase activity determination was prepared by digesting casein for 2 days in this manner with the most active pancreatic enzyme preparation tested. For these assays, 20 mg. of the crude peptidase preparation were incubated at pH 7 for 4 hours with the pancreatic digest of 1 gm. of casein. Pepsin (Difco), whole pancreas (Viobin), and erepsin (Difco) were selected for this work. By employing these enzymes successively for short periods of incubation, the rates of the digestion of the raw and heated casein samples were determined with periodic α-amino nitrogen and microbiological amino acid determinations.

10 gm. each of the raw and the two heated casein samples were placed in 2 liter Erlenmeyer flasks with 500 ml. of 0.1 N hydrochloric acid and shaken at 37° overnight. 10 ml. of enzyme solution containing 50 mg. of pepsin (Difco) were then added to each flask. A fourth flask containing 500 ml. of 0.1 N hydrochloric acid and 50 mg. of pepsin, but no substrate, served as a blank. After 40, 70, and 100 minutes, pH measurements were made and 2 ml. aliquots were removed from each flask for Van Slyke α-amino nitrogen determinations, to measure the progress of the digestion. At 100 minutes, the rate of digestion was decreasing rapidly; therefore, at 120 minutes a 50 ml. aliquot was removed from each flask. These aliquots were heated in a boiling water bath for 15 minutes to inactivate the pepsin, and were stored at −4° for amino acid assays.

Immediately after removal of the 50 ml. aliquots, 8 ml. of 5 N sodium hydroxide were added to each digestion flask to neutralize the solutions partially and to arrest the peptic activity. The solutions were then adjusted to pH 8.2 with 5 N sodium hydroxide and 10 ml. of toluene added. 10 ml. of pancreas (Viobin) solution (filtered water extract containing 10 mg. per ml.) were then pipetted into each flask. The course of the digestion was again followed with pH measurements and Van Slyke α-amino nitrogen determinations; after 1 hour the solutions were readjusted to pH 8.2. After 2 hours 50 ml. aliquots were removed and treated as before.

The contents of each flask were then adjusted to pH 7.0 and 10.0 ml. of erepsin (Difco, filtered water extract containing 5 mg. per ml.) were immediately added. The progress of digestion was again determined by periodic pH measurements and α-amino nitrogen determinations. After 2 hours, 50 ml. aliquots were removed and treated as above. The digestion was allowed to continue for 5 days longer, at which time 50 ml. aliquots were again removed.

Amino Acid Assays—Sixteen amino acids were determined microbiologically on the acid and enzyme hydrolysates with the following organisms for the amino acids indicated: *Lactobacillus arabinosus* 17-5 for glutamic acid, leucine, tryptophan, valine, and phenylalanine; *Leuconostoc mesenteroides* P-60 for aspartic acid, cystine, glycine, histidine, isoleucine, lysine, proline, and tyrosine; *Streptococcus faecalis* R for methionine and threonine; and *Lactobacillus delbrueckii* 3 for arginine. All amino acids except cystine were determined by the methods of Henderson and Snell (8). Cystine was determined with an oxidized peptone medium as described by Riesen *et al.* (9).

RESULTS AND DISCUSSION

Amino Acid Content of Raw versus Heated Casein—The data presented in Table I show that, with the exception of cystine, the amino acid content of casein as measured microbiologically after acid hydrolysis (alkaline hy

TABLE I

*Liberation of Microbiologically Available Amino Acids from Raw and Heated Casein by Acid and Enzymes**

| Amino acid | Amino acid content of acid hydrolysates† | | | | Percentage liberation by digestive enzymes‡ | | | | | | | | | | | | |
| | | | | | Pepsin, 2 hrs. | | | Pepsin + pancreas, 2 hrs. each | | | Pepsin + pancreas + erepsin, hrs. each | | | Same, continued 5 days | | |
	Raw	4 min §	20 hrs §	Average	Raw	4 min.§	20 hrs.§	Raw	4 min.§	20 hrs.§	Raw	4 min §	20 hrs.§	Raw	4 min.§	20 hrs.§
	per cent	per cent	per cent	per cent												
Arginine	3.20	3.18	3.14	3.17	40	46	28	81	81	58	96	98	72	97	107	89
Aspartic	6.54	6.34	6.17	6.35	1	1	6	1	16	6	5	4	7	11	12	6
Cystine‖	0.50	0.52	0.14	0.51‖	0	1	2	1	3	7	10	7	21	60	40	57
Glutamic	19.27	19.07	18.95	19.10	5	5	3	22	24	17	51	52	36	106	116	83
Glycine	1.69	1.56	1.71	1.65	4	0	4	9	9	9	20	15	16	56	113	48
Histidine	2.60	2.51	2.49	2.54	0	0	1	2	2	5	7	8	8	37	47	34
Isoleucine	5.34	5.61	5.42	5.46	0	0	1	3	3	5	8	8	7	40	49	29
Leucine	8.44	8.61	8.81	8.62	11	9	5	41	44	34	57	58	39	87	99	78
Lysine	6.54	6.50	6.33	6.49	1	0	1	10	10	7	35	33	20	81	81	44
Methionine	2.13	2.11	2.26	2.17	4	4	13	55	59	50	85	90	49	146	150	94
Phenylalanine	4.70	4.49	4.65	4.61	10	13	4	46	48	48	63	61	48	85	119	77
Proline	10.69	9.56	9.34	9.86	0	0	1	1	1	1	1	1	2	19	22	17
Threonine	3.58	3.69	3.50	3.59	3	3	6	27	28	19	44	44	26	83	88	48
Tryptophan¶	1.03	1.09	1.03	1.05	10	12	9	56	61	50	82	87	62	97	100	85
Tyrosine¶	4.51	4.19	4.38	4.36	1	1	1	36	44	31	57	50	35	82	81	77
Valine	6.72	6.62	6.68	6.67	2	2	3	18	20	16	41	49	31	113	132	86
α-Amino N**	73††	76††	77††		9	11	10	19	21	17	27	30	24	50	52	40
Amino acid totals‡‡	87.48	85.77	85.00	86.20	5	5	5	22	24	19	39	40	27	77	85	59

* The amino acid figures represent the average of from three to five microbiological assays of two different hydrolysates. The total nitrogen in the raw casein was 13.51, 4 minute casein 13.66, and the 20 hour casein 13.50 per cent; the moisture contents were 8.00, 8.14, and 6.50 per cent, respectively.

† See the text for the conditions of hydrolysis. The acid hydrolysis values are expressed as per cent (gm. per 100 gm. of protein) amino acid yielded by casein; the values for the heated caseins were adjusted to the nitrogen content of the raw casein.

‡ See the text for the conditions of enzyme hydrolysis. The amino acid values were computed by dividing the amount of amino acid liberated by enzymatic hydrolysis by the amount liberated from casein by acid hydrolysis (average of raw and heated caseins) and multiplying by 100. The enzyme α-amino nitrogen figures were computed similarly.

§ The casein was heated by autoclaving for 4 minutes or 20 hours at 15 pounds.

‖ The acid hydrolysis average figure was computed from the liberation from the raw and 4 minute heated casein. Enzymatic liberation values for 20 hour heated casein were computed with the acid liberation figure for this casein sample instead of the average figure.

¶ Since alkaline hydrolysis was used, complete racemization was assumed and figures represent twice the actual amounts measured.

** Determined by the semimicro nitrous acid method of Van Slyke.

†† The figures represent the percentage of the total nitrogen released in the Van Slyke procedure.

‡‡ Acid hydrolysis totals are the sums of the percentages of the amino acids liberated. Enzyme hydrolysis totals were obtained by dividing the total amount of microbiologically available amino acids liberated enzymatically by the total amount liberated from the corresponding casein sample by acid hydrolysis (or alkaline hydrolysis) and multiplying by 100.

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sis for tyrosine and tryptophan) was not affected significantly by autoclaving at 15 pounds pressure (121°) for 4 minutes or 20 hours. The cystine value was reduced to one-fourth that of the raw casein by autoclaving for 20 hours, but was unchanged by autoclaving for 4 minutes.

Liberation of Amino Acids by Digestive Enzymes—In Fig. 1 are shown typical hydrolysis curves of raw casein and casein autoclaved for 4 minutes and 20 hours at 15 pounds when subjected to successive digestion with pepsin, pancreas enzymes, and erepsin. Heated casein was digested more
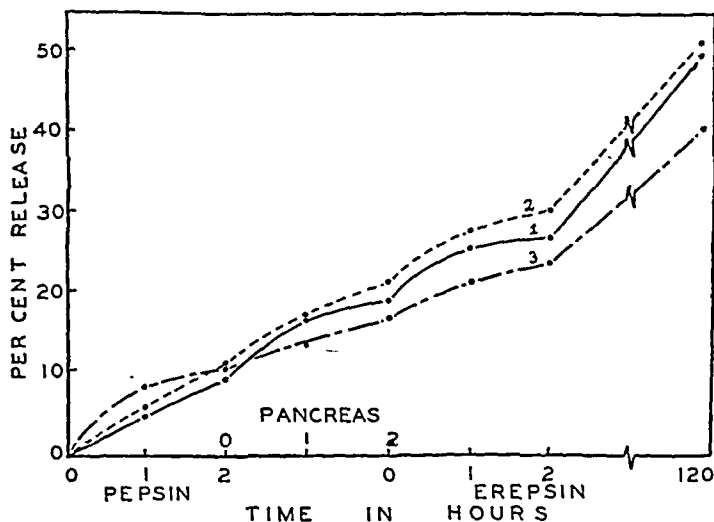

FIG. 1. Release of α-amino nitrogen from raw casein (Curve 1), casein autoclaved 4 minutes (Curve 2), and casein autoclaved 20 hours (Curve 3) by successive digestion with pepsin, pancreas enzymes, and erepsin. The digests were adjusted to pH 1.0 for peptic digestion, to pH 8.0 for pancreatic digestion, and to pH 6.8 for ereptic digestion. There were insignificant changes in pH during the course of the digestion, except after 1 hour with pancreas enzymes when the raw casein digest was pH 6.4, 4 minute casein pH 5.9, and the 20 hour casein pH 7.1. The contents were readjusted to pH 8 for the remainder of the pancreatic digestion.

rapidly in the initial stages by pepsin; the extent of digestion was about the same at the end of 2 hours, regardless of the heat treatment. The rate of release of α-amino nitrogen during pancreatic and ereptic digestion of casein was slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. A disproportionately large reduction in pH in relation to the release of α-amino nitrogen occurred during the 1st hour of pancreatic digestion.

The interpretation of the data on the extent of amino acid liberation by enzymes when measured by microbiological procedures is complicated by

the probable utilization of peptides by the microorganisms commonly used for the assay of amino acids. Peptides that have been investigated thus far show variable activity, ranging from 0 to 100 per cent when assayed for the amino acids which they contain (10–13). In view of this variation in response of microorganisms to peptides, the term "microbiologically available" amino acids will be used in the discussion of Table I. The above objection invalidates these values for other than gross comparative purposes.

Pepsin Digestion

The liberation of α-amino nitrogen and microbiologically available amino acids from casein by pepsin was unaffected by heat treatment. There was considerable variation in the extent of liberation of the individual amino acids; a relatively large percentage of the arginine and much lower percentages of glutamic acid, glycine, leucine, methionine, phenylalanine, threonine, and tryptophan were released. No significant quantities of the other amino acids were liberated. The percentage of arginine that became microbiologically available was about 8 times as great as the average[1] percentage of all sixteen amino acids. Half of the α-amino nitrogen liberated (measured by the Van Slyke method) could be accounted for by microbiologically available amino acids.

Pepsin Plus Pancreas Digestion

The liberation of α-amino nitrogen and the "average" liberation of mino acids from casein by pepsin followed by pancreas were slightly increased by autoclaving for 4 minutes and decreased by autoclaving for 20 hours. All amino acids except proline were released by pancreas enzymes. The extent of liberation of aspartic acid, cystine, histidine, and isoleucine was less than 10 per cent, while the total amino acid liberation was approximately 20 per cent and was equal to the percentage release of α-amino nitrogen.

The percentage of the arginine which became microbiologically available was approximately 4 times as great as the average of the other amino acids. Hunter and Dauphinee (14) have also reported a rapid cleavage of this amino acid from casein and gelatin by trypsin. It is not known, however, whether arginine is liberated in the form of peptides having high activity for *Lactobacillus delbrueckii* 3 or as free arginine.

In general the results obtained with pancreas enzymes agree with those of other workers. Abderhalden (15) found more rapid liberation of tyrosine than of glutamic acid from casein by pancreatin. Hunter (16) found that a proline fraction exists in casein which is comparatively resistant to tryptic

[1] See Table I, foot-note ‡‡.

digestion. It is possible that the low values obtained in the present study with enzyme digests are due to the absence of proline-releasing enzymes in the preparations used.

Digestion with Pepsin, Pancreas, and Erepsin

The extent of liberation of microbiologically available amino acids from casein was determined after following the pepsin-pancreatic digests prepared in the above manner with digestion by erepsin for 2 hours and 5 days. The extent of liberation of aspartic acid, cystine, histidine, isoleucine, and proline after 2 hours was below 10 per cent, while the total liberation of amino acids was about 40 per cent. Arginine, methionine, and tryptophan were nearly entirely liberated. After 5 days, most amino acids were completely available to the microorganisms used for assay. The liberation of cystine, which had been low throughout the digestion, increased considerably after 5 days. The release of aspartic acid and proline remained low, i.e. 10 to 20 per cent, while the liberation of methionine was approximately 150 per cent. The latter result could indicate activity of peptides above that expected on the basis of methionine content, or destruction of methionine during acid hydrolysis. The values for acid hydrolysates reported here are lower than those obtained by many other workers, a result which supports the latter explanation. In a previous study, an average value of 2.69 ± 0.26 per cent in the dried, ash-free protein was reported, while six values cited from the literature averaged 2.85 ± 0.21. The value for raw casein reported in Table I is 2.33 corrected for moisture and ash.

The amino acid totals exceeded the α-amino nitrogen values after digestion by erepsin for 2 hours or for 5 days. The average peptide size at the end of 5 days was 2 amino acid residues.

It should be pointed out that the extent of liberation of glutamic acid from proteins by enzymes is not strictly comparable to that obtained on acid hydrolysates, since in enzyme hydrolysates any glutamine released would have been converted to pyrrolidonecarboxylic acid by the heating to inactivate the enzymes, and the subsequent sterilization of the assay tubes by autoclaving. According to Hamilton (17) glutamic acid is relatively stable to acid hydrolysis and to autoclaving, whereas glutamine is converted to glutamic acid when heated below pH 3 and to the inactive pyrrolidonecarboxylic acid during autoclaving at pH 6.5. Work in this laboratory has shown that sterilization of the medium by autoclaving for 10 minutes at 12 pounds pressure at neutral pH causes complete or nearly complete loss of activity of free glutamine for *Lactobacillus arabinosus*. It appears from Table I that glutamic acid was quantitatively liberated by enzymes in 5 days or less. This may be accounted for by assuming (*a*) deamidation of the glutamine during enzyme hydrolysis, (*b*) release of

glutamine in peptide combinations which are not cyclized by heat, or (c) release of peptides of glutamic acid possessing more activity than glutamic acid itself, thus compensating for the glutamine destroyed during autoclaving.

The liberation of aspartic acid, in contrast to glutamic acid, was low throughout the 5 day digestion period. Whereas glutamine is fully as active as glutamic acid for *Lactobacillus arabinosus* (18), asparagine is much less active than aspartic acid for *Leuconostoc mesenteroides* P-60 (19). The low values obtained may be due to the liberation in the form of asparagine or peptides.

Casein appears to be less affected by heat than soy bean protein. Very little increase in digestibility was noted after 4 minutes of autoclaving; some destructive effects occurred after 20 hours. In both soy bean and casein, lysine was among the amino acids whose rate of release by enzymes after heat treatment was affected most adversely.

SUMMARY

1. Autoclaving casein at 15 pounds pressure for 4 minutes had no effect on the amino acid composition as measured by microbiological determinations after acid hydrolysis. Autoclaving for 20 hours reduced the cystine content, but did not affect the amounts of other amino acids.

2. The rate of release of α-amino nitrogen and of microbiologically available amino acids during a 2 hour digestion of casein with a limited quantity of pepsin was unaffected by the heat treatments. The release of amino acids from the pepsin digests after treatment with desiccated pancreas and then with erepsin was higher in the casein autoclaved for 4 minutes and lower in casein autoclaved for 20 hours, compared to unheated casein.

3. The release of amino acids from raw casein by pepsin was 5 per cent with 10 per cent liberation of α-amino nitrogen. 40 per cent of the arginine became microbiologically available.

4. Pancreatic digestion for 2 hours released approximately 22 per cent of the amino acids and 19 per cent of the α-amino nitrogen and, after an additional 2 hour digestion with erepsin, 39 per cent of the amino acids was available to the lactic acid bacteria and 27 per cent of the α-amino nitrogen was released. Continued digestion with no additional enzymes for 5 days released 50 per cent of the α-amino nitrogen and 77 per cent of the amino acids in microbiologically available form. Only small amounts of aspartic acid and proline were liberated.

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cooled and centrifuged and the supernatants poured into 15 ml.
ifuge tubes, diluted to about 8 ml., and treated with 6 drops of con-
ated HCl and 4 ml. of 5 per cent tannic acid as above. The precipi-
separated from the above supernatants were shaken an hour with 8
if 0.5 N HCl, centrifuged, and retreated with 0.5 N HCl. The super-
nts from both treatments with 0.5 N HCl were joined and precipitated
8 ml. of 5 per cent tannic acid. The tannic acid precipitates, the
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c acid in a total volume of at least 6 ml. and continued for 12 hours with
nized Hengar granules. The distillation and titration were carried out
ording to Ma and Zuazaga (4). The average total nitrogen in the muscle
quots was about 10 mg. per aliquot, in the pooled fascia samples 0.2 mg.
pooled sample, and in the bone aliquots 2 mg. per aliquot.

Results—Table I includes all results not discarded for known errors in
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uble non-collagen nitrogen and bone total nitrogen are not included.
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yses, 0.03 mg. of N in the bone analyses, and 0.01 mg. of N in the
es. The percentage of total nitrogen in the acid-soluble frac-
y constant in all three tissues. Deviations from the mean
ent for muscle, 24 per cent for fascia, and 14 per cent for
lightly higher values in the youngest animals, lie within
mental error. In all three tissues, therefore, the collagen
nsoluble non-collagen nitrogen constitute about 80 to
total nitrogen. (For example, the figures for muscle
d acid-insoluble non-collagen nitrogen are 10 and 70
y for Animal 5, and 50 and 30 per cent respectively for

rogen in the bone fractions agreed with the total nitro-
dently, with an average deviation of 3 per cent and
er cent. An exception was the analysis of Animal
cent deviation is attributable to an error in the total
h explains the low collagen nitrogen expressed as
ig t when compared with the analysis of Animal 13.
from the normal values of bone collagen nitrogen in
the youngest animal and appreciable in the middle
is no effect of fasting in these groups, the effect of

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were cooled and centrifuged and the supernatants poured into 15 ml. centrifuge tubes, diluted to about 8 ml., and treated with 6 drops of concentrated HCl and 4 ml. of 5 per cent tannic acid as above. The precipitates separated from the above supernatants were shaken an hour with 8 ml. of 0.5 N IICl, centrifuged, and retreated with 0.5 N HCl. The supernatants from both treatments with 0.5 N HCl were joined and precipitated with 8 ml. of 5 per cent tannic acid. The tannic acid precipitates, the supernatants overlying them after centrifugation, and the material not rendered soluble by HCl were treated as were the similar fractions from muscle. Other aliquots of the bones were analyzed for total nitrogen.

The method outlined is a micromodification of the method of Spencer, Morgulis, and Wilder (2). The digestion in the nitrogen analyses was begun with 2.0 ml. (4.0 ml. for non-collagen nitrogen) of concentrated sulfuric acid in a total volume of at least 6 ml. and continued for 12 hours with selenized Hengar granules. The distillation and titration were carried out according to Ma and Zuazaga (4). The average total nitrogen in the muscle aliquots was about 10 mg. per aliquot, in the pooled fascia samples 0.2 mg. per pooled sample, and in the bone aliquots 2 mg. per aliquot.

Results—Table I includes all results not discarded for known errors in technique, except that the separate figures for acid-soluble and acid-insoluble non-collagen nitrogen and bone total nitrogen are not included. The average deviation from the mean is about 0.1 mg. of nitrogen in the muscle analyses, 0.03 mg. of N in the bone analyses, and 0.01 mg. of N in the fascia analyses. The percentage of total nitrogen in the acid-soluble fraction was relatively constant in all three tissues. Deviations from the mean values of 20 per cent for muscle, 24 per cent for fascia, and 14 per cent for bone, except for slightly higher values in the youngest animals, lie within the range of experimental error. In all three tissues, therefore, the collagen nitrogen and acid-insoluble non-collagen nitrogen constitute about 80 to 85 per cent of the total nitrogen. (For example, the figures for muscle collagen nitrogen and acid-insoluble non-collagen nitrogen are 10 and 70 per cent respectively for Animal 5, and 50 and 30 per cent respectively for Animal 11.)

The sum of the nitrogen in the bone fractions agreed with the total nitrogen determined independently, with an average deviation of 3 per cent and no deviation over 8 per cent. An exception was the analysis of Animal 13, in which a 20 per cent deviation is attributable to an error in the total nitrogen analysis, which explains the low collagen nitrogen expressed as per cent of dry bone weight when compared with the analysis of Animal 13.

In Table I the drop from the normal values of bone collagen nitrogen in scurvy is marked in the youngest animal and appreciable in the middle age group. As there is no effect of fasting in these groups, the effect of

TABLE I

Variations in Collagen Distribution (Experiment 1)

Animal No.	Age	Diet		Weight during experiment		Collagen N, per cent of total N			Collagen N, per cent of dry bone weight
		Nature	Duration	Initial	Final	Muscle	Fascia	Bone	
	days		days	gm.	gm.				
1	10	Adequate	22	131	277	10 10 10 14	76 61 58	51	2.35
2	10	"	22	138	286	9 9 10 10	61 37	54 48	2.50
3	37	"	25	302	472	13 12 12 11	66 60 70	64 61	2.52
4	37	"	25	326	502	12 14 12 14	74 62 71	60 60	2.64
5	90	"	29	466	643	11 11 9 10	72 61 54	70	2.62
6	90	"	29	459	631	10 10 10 7	50 56 67	68 66	2.58
7*	10	" No food	15 7	116	120	55 53 53 53	57 48 58	52 48	2.65
8	10	Adequate No food	15 7	127	152	57 58 56 57	39 40 48	57.5 57	3.16
9	37	Adequate No food	16 9	302	256	55 73 66 67	67	66	2.71
10*	37	Adequate No food	16 9	326	262	60 54 59 53		68 67	2.90

TABLE I—*Concluded*

Animal No.	Age	Diet		Weight during experiment		Collagen N, per cent of total N			Collagen N, per cent of dry bone weight
		Nature	Duration	Initial	Final	Muscle	Fascia	Bone	
	days		days	gm.	gm.				
11*	90	Adequate	11	523	365	41	64	58	2.38
		No food	11			51	84	57	
						51			
						49			
12	90	Adequate	11	679	465	13	68	62	2.20
		No food	11			19	78	60	
						14	75		
						14			
13	10	Scorbutic	22	108	146	16	62	37	1.77
						17	66	32	
						13			
13†								33	2.01
								33	
14	10	"	22	108	146	20	57		
						22	92		
						24	62		
						24			
15	37	"	25	348	365	22	35	54	2.43
						20	62	52	
						19	73		
						13			
16	37	"	25	343	334	13	56	50	2.35
						15	62		
						18			
						18			
17	90	"	29	598	577	16	72	73	2.56
						18	72	74	
						19			
						15			
18	90	"	29	610	420	11	68	68	2.68
						10	79	67	
						13	64		
						11			

* Died.

† Left femur of Animal 13.

scurvy is not to be ascribed to inanition. The drop is consistent with the histological findings in scorbutic bone, an interruption of the conversion of precollagenous material to collagen. An increase in the collagen per cent with age and a decreased effect of scurvy with age are observable by an inspection of the data and are statistically significant. The constancy of

the collagen nitrogen expressed as per cent of dry bone weight in normal, fasted, and older scorbutic animals is to be noted.

In muscle the direction (rise) of the changes in the collagen nitrogen is the same in both fasting and scurvy and the rise is greater in fasting. Hence the rise in scurvy may be attributed to inanition.

The fascia analyses are obviously at the lower limit of useful precision of the method in its present form. The average collagen values for normal, fasting, and scorbutic animals are 62, 61, and 66 per cent respectively; for the youngest, intermediate, and oldest animals, 58, 64, and 70 per cent respectively, the probable error being about 2 per cent for each average. There is a significant increase in collagen per cent with age. It

TABLE II

Collagen and Total Nitrogen Content of Gastrocnemius (Experiment 2)

Animal No.	Diet		Weight during experiment		Gastrocnemii, right and left combined				
	Nature	Dura-tion	Initial	Final	Weight	Colla-gen N	Total N	$\dfrac{\text{Collagen N}}{\text{Total N}} \times 100$	$\dfrac{\text{Collagen N}}{\text{Animal weight}}$
		days	gm.	gm.	gm.	mg.	mg.		mg. per kg.
A	Water	8	440	310	2.30	12.0	77.1	15.6	38.7
B	"	9	430	240	1.30	11.4	45.7	24.9	47.5
C	Adequate	9	400	440	2.95	11.1	98.3	11.3	25.2
D	"	9	400	480	2.69	11.2	90.6	12.4	23.3
E	"	0		410	2.42	8.65	75.4	11.5	21.1
F	"	0		380	2.38	8.62	75.9	11.4	22.7

is demonstrably improbable that scurvy produces a considerable (5 per cent) decrease in collagen percentage at all ages.

Experiment 2

Six growing guinea pigs were used. Four were of approximately equal weight at the start of the experiment and, of these, two were continued on a normal diet and two received water but no food from the start of the experiment. One of the latter died on the 8th day of the experiment and the remaining three, together with two animals having approximately the same weight that the original four had at the start of the experiment, were killed at the same time, 9 days after the start. From each animal both gastrocnemius muscles *with tendons and investing fascia* were removed and weighed. Collagen and total nitrogen were determined by the general method of Lowry, Gilligan, and Katersky, except that nitrogen analyses replaced the weighings. The results are given in Table II.

Experiment 3

A 420 gm. guinea pig was rapidly dissected into four fractions: skin, large muscles and fascia, bones and adherent muscles and fascia, and organs. The four fractions were weighed and analyzed as in Experiment 2, except that the "bone and adherent muscle and fascia" fraction was not ground fine but extracted for 3 days with 0.1 N NaOH (instead of the usual fine grinding and 14 hour extraction). At the end of 3 days the tissue was almost completely colorless. Neutralization and autoclaving of this fraction were completed and the undissolved bone and fiber (dry weight, 14.5 gm.) were analyzed separately for total nitrogen (188 mg.), this nitrogen being assigned arbitrarily half to the collagen and half to the non-collagen nitro-

TABLE III

Distribution of Collagen (Experiment 3)

	Weight	Collagen N	Total N
	gm	*mg*	*mg.*
Whole animal .	420		
Skin, shaved	61	1243	3361
Large muscles, tendon, and fascia	106	270	669
Bones, adherent muscles, and fascia	78	942	2359
Organs...	80	88	1507
Hair	15		
Feces	42		
Shed blood .	35		1000*
Hairless animal	360	2543	8896

Total collagen nitrogen = 29 per cent of total nitrogen.

* Estimated.

gen of the "bone and adherent muscle and fascia" fraction. Shaved hair from the skin, the feces, and the blood shed during the dissection were collected separately, and the nitrogen content of the blood was calculated. All nitrogen analyses were done in duplicate and the figures in Table III are subject to errors of not greater than 2 or 3 per cent arising from variation in the nitrogen analyses.

DISCUSSION

The analyses summarized in Table II show that in the fasted animal at the growing age collagen continues to be synthesized at about the same rate as in the well fed animal. The absolute values for collagen nitrogen estimated by the different methods employed in Experiments 1 and 2 are not more than approximately comparable (3), and the 2-fold increase in colla-

gen nitrogen during fasting, expressed as per cent of total nitrogen observed in Animal B, Table II, represents the relative increase in collagen in a whole muscle including tendon and investing fascia, while the 4-fold increases during starvation in Table I occur in samples from which tendon and fascia have been removed. The results by the two methods are not therefore necessarily in disagreement.

The analysis summarized in Table III shows that the fascia contains a large fraction of the total collagen of the guinea pig, and the large amount of fascia in the thoracic wall was made strikingly apparent when the crudely dissected skeleton was stirred with 0.1 N NaOH. The analysis of the whole animal has not the precision attained with smaller samples which are more carefully ground, but it clearly demonstrates that collagen formation is quantitatively the most important single reaction leading to the sequestration of nitrogen for growth.

Collagen is localized in tissues which are primarily concerned with the maintenance of the body's gross form against mechanical pressures arising within the body (e.g., muscle tonus, blood pressure) and balanced partially by forces in the environment (e.g., the pressure of gravity on the muscles, tendons, and bones; the atmospheric pressure). The growth of such structures is a geometrical extension of the tissues against the pressures exerted upon them by the environment and is a characteristically biological evasion of the principle of Le Chatelier. The observation of Wolbach and Howe (1) that blood vessels fail to grow into wounded areas produced in scorbutic guinea pigs may be of interest in this respect.

SUMMARY

A micromethod for the determination of collagen in bone is described. The distribution of collagen in the guinea pig and the effect of age and fasting on the muscle collagen of guinea pigs has been investigated.

Collagen nitrogen constitutes a considerable fraction of the total body nitrogen, and during growth collagen appears to be synthesized in fasting animals at about the same rate as in non-fasting animals.

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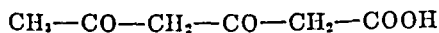
SYNTHESIS AND PROPERTIES OF TRIACETIC ACID*

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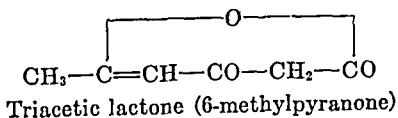
Advances in intermediary fat metabolism have been restricted considerably by the lack of postulated intermediate compounds. Triacetic acid (β,δ-diketohexanoic acid) would be the product of multiple alternate oxidation of hexanoic acid, and would also be the first product resulting from the condensation of acetoacetic acid with acetate by a mechanism postulated for fatty acid synthesis (1). Triacetic lactone, loosely referred to as "triacetic acid" by Breusch and Ulusoy (2, 3), has been used in metabolic experiments by these investigators, but to the authors' knowledge free triacetic acid has not been isolated or tested for biological activity.

The compounds under principal consideration are represented in the accompanying formulas.

$$CH_3—CO—CH_2—CO—CH_2—COOH$$
Triacetic acid (β,δ-diketohexanoic acid)

$$CH_3—C=CH—CO—CH_2—CO$$
(with O bridging the first C and the last CO)
Triacetic lactone (6-methylpyranone)

$$CH_3—CO—CH_2—CO—CH_2—COOC_2H_5$$
Ethyl triacetate
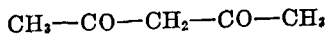
$$CH_3—CO—CH_2—CO—CH_3$$
Acetylacetone

In this paper the synthesis of triacetic acid is described, involving the following series of reactions: dehydroacetic acid → triacetic lactone → copper ethyl triacetate → ethyl triacetate → triacetic acid. Triacetic lactone was condensed with ethyl alcohol in a sealed tube to break the stable enol ring, with formation of ethyl triacetate, and the latter was isolated as the stable copper salt by a modification of Sproxton's procedure (4). Isolation of the free acid involved prior study of the conditions of esterification to preserve the unstable acid. Properties of the free acid, including its color reaction with o-phenylenediamine and its catalytic

decarboxylation with various aromatic amines, are reported. Analytical data on the free acid and its 2,4-dinitrophenylhydrazine derivatives are also given.

Methods

In order to determine the proper conditions for hydrolysis of triacetic ester and to compare the properties of triacetic acid with acetoacetic acid, catalytic decarboxylation by aromatic amines was employed. In this method triacetic ester and lactone are not decarboxylated, while the free acid is readily attacked. Decarboxylation methods with aniline citrate (5), aniline acetate-sodium cyanide (6), o- and p-phenylenediamine citrates (7), as well as modifications of these methods were variously used. From 3 to 8 micromoles of the β-keto acids were employed in a 3 ml. volume in Warburg flasks, and the catalysts added from the side arm after appropriate equilibration of the reaction mixtures at 30°. The flasks were filled with nitrogen when phenylenediamine catalysts were employed.

The o-phenylenediamine color reaction described in the following paper (8) was also employed in controlling the isolation of triacetic acid. This reaction is given directly by triacetic acid, triacetic ester, and acetylacetone which contain a β-diketone group ($-CH_2-CO-CH_2-CO-CH_2-$). Triacetic lactone does not react directly but can be converted to acetylacetone by acid hydrolysis.

Solutions of acetoacetic acid used in these studies were prepared according to the method of Krebs and Eggleston (5).

Synthesis of Triacetic Acid

Triacetic Lactone—Triacetic lactone was prepared from dehydroacetic acid (Eastman) according to the method described by Collie (9). The product had a melting point of 186–187° (Collie, 187–188°) and a neutral equivalent of 130 (Collie, 130; theoretical 126).

Copper Ethyl Triacetate—This substance was previously isolated by Sproxton (4). A mixture of 7.56 gm. (0.06 mole) of triacetic lactone and 80 to 90 ml. of absolute ethyl alcohol was heated in a dry sealed tube at 110° for 48 hours. After cooling, the contents were diluted to 100 ml. with ethyl alcohol and analyzed for diketones (8). Analysis showed that 43 per cent of the lactone remained unchanged, and 47 per cent was present as ethyl triacetate, a total of 90 per cent of the original lactone being accounted for. No reaction took place at 100°, and heating for more than 48 hours did not increase the yield.

To the alcohol solution were added 0.5 mole of copper acetate and a slight excess of ammonia for each mole of ethyl triacetate found (in the above case 0.014 mole of copper acetate as a 0.3 M aqueous solution and

0.03 mole of ammonia as concentrated ammonium hydroxide). After 2 hours standing at room temperature the gray-blue precipitate was collected, washed with cold ethyl alcohol, and dried over P_2O_5 *in vacuo*. The yield was 3.3 gm. or 58 per cent of the copper salt calculated to be present in the solution. The melting point was 182.5–183.5°. Sproxton (4) reported 183–184°.

No precipitate formed in the absence of ammonia, the yield was lowered if a greater excess of ammonia was added, and only a small amount of impure product was obtained if the mother liquor was cooled to 5° or concentrated *in vacuo* to a small volume.

Ethyl Triacetate Solution—200 to 400 mg. of copper ethyl triacetate were finely suspended in 5 ml. of water and H_2S passed through the ice-cold solution for 6 hours. The mixture was filtered, diluted to 10 ml., and aerated to remove excess H_2S. Diketone analysis indicated a 97 to 100 per cent yield of ethyl ester.

Isolation of Triacetic Acid—To the 10 ml. of triacetic ester solution (0.1 to 0.2 M) resulting from the H_2S decomposition of copper ethyl triacetate was added 1 N sodium hydroxide in the ratio of 2 moles of alkali for each mole of triacetic ester present. After standing at 5° for 24 hours, the solution was centrifuged and the clear supernatant liquid acidified to Congo red with 1 N sulfuric acid. The solution was then extracted three times with equal volumes of redistilled ethyl ether, the combined ether extracts washed with 0.1 volume of water, dried with anhydrous sodium sulfate for 1 hour, and finally evaporated in a stream of air. The oily residue was placed in a vacuum desiccator over sulfuric acid at 5°. After 3 to 4 days a light yellow, waxy solid, consisting of rhombic plates, appeared. The triacetic acid yield was 40 to 50 per cent of the theoretical calculated from the copper ethyl triacetate used.

After recrystallization from ethyl ether-petroleum ether, triacetic acid had a melting point of 29–31°, a neutral equivalent of 147 (theory for $C_6H_8O_4$, 144), and a pK of approximately 3.3. The acid gave the following analysis.

$C_6H_8O_4$. Calculated, C 50.0, H 5.55; found, C 49.2, H 5.55

The 2,4-dinitrophenylhydrazine derivatives of triacetic acid were prepared by the general method of Clift and Cook (10) as follows: To a 0.04 M solution of triacetic acid was added an equimolar amount of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. After standing overnight at 5°, the precipitate was dissolved in saturated Na_2CO_3. The light brown solution was acidified at 5° with concentrated HCl, and the yellow derivative which separated melted at 155–182°. Upon solution in ethyl acetate, this material was separated into two components by precipi-

tation with petroleum ether. With 2 volumes of petroleum ether, yellow hexagonal crystals separated which had a melting point of 189–191° (with gas evolution). 4 volumes of petroleum ether precipitated yellow needle-like crystals, with a melting point of 149–150° (with gas evolution). After drying the derivatives *in vacuo* over H_2SO_4, their neutral equivalents were determined by the method of Clift and Cook (10). The method yielded theoretical values with acetoacetic acid, with brom-thymol blue as the indicator. Nitrogen analyses by the micro-Dumas method were also made, with the following results.

$C_{12}H_{12}O_7N_4$.	Calculated.	N 17.28,	neutral equivalent	324
M.p. 189–191°. Found.	"	" 17.86	" "	326
" 149–150°. "		" 17.74	" "	324
$C_{12}H_{10}O_6N_4$.	Calculated.	" 18.30	" "	306
M.p. 189–191°. Found.	"	" 17.86	" "	326
" 149–150°. "		" 17.74	" "	324

Both derivatives thus had the same neutral equivalent and nitrogen content. Neither gave a Knorr test (11) for the pyrazoline ring after reduction with sodium in ethyl alcohol. The neutral equivalent and negative Knorr test indicate an open chain 2,4-dinitrophenylhydrazone, although the nitrogen analysis falls between the theoretical values for an open chain compound and a ring structure.

Properties of Triacetic Ester

Dilute aqueous solutions (0.02 to 0.1 M) of ethyl triacetate are light yellow in color and have a sweet ester-like odor. Based on diketone content, such solutions at pH 4 to 5 are completely stable at 5° for a week, and are 10 per cent decomposed in 3 weeks. At 28° ethyl triacetate solutions (0.005 to 0.01 M) are stable for 16 hours at pH 4.5 but are 35 to 40 per cent decomposed at pH 7.2. Triacetic acid, triacetic lactone, or acetoacetic acid did not appear to be products of the decomposition.

o-Phenylenediamine Reaction—Triacetic ester reacts directly with *o*-phenylenediamine and hence must have a β-diketone group. In the direct reaction with *o*-phenylenediamine the ester yields 80 to 82 per cent of the color obtained from equimolar amounts of acetylacetone or triacetic acid, and an initial lag in color development is noted. After distillation from 2.5 N H_2SO_4, the lag in color development is no longer noted and the final color intensity is equivalent to that of acetylacetone.

Saponification of Ethyl Triacetate—For the isolation of triacetic acid, a rather extensive preliminary study was necessary to determine the proper conditions for the hydrolysis of ethyl triacetate, since β,δ-diketones, including acetylacetone (12), are readily split by alkali. For proper interpretation of later metabolic experiments it was therefore necessary to rule

out the presence of products other than triacetic acid, and to isolate the latter rather than to assume its presence after hydrolysis, as is commonly done with more stable compounds.

To evaluate the results of different methods of saponification, both the catalytic decarboxylation method and the o-phenylenediamine color reaction were employed. Since the ester is not decarboxylated, the appearance of β-keto acids can be determined by the decarboxylation reaction, and failure to obtain equivalent amounts of β,δ-diketones by the color reaction was assumed to be due to side reactions involving loss of the diketone group. Acetylacetone gives the color reaction but cannot be

TABLE I

Saponification of Triacetic Ester

"Free" diketone determined by direct o-phenylenediamine color reaction; "total" diketone after acid hydrolysis (8). CO_2 determined by the aniline citrate decarboxylation method. Amount of alkali present, 2 moles for each mole of triacetic ester used.

Triacetic ester ("total" diketone)	Hydrolysis temperature	Hydrolysis time	"Free" diketone after hydrolysis	Diketone loss	CO_2	Ratio	
						CO_2 to "free" diketone after hydrolysis	CO_2 to initial "total" diketone
micromoles	*°C.*	*hrs.*	*micromoles*	*per cent*	*micromoles*		
910	30	18	615	32	660	1.07	0.73
91	30	18	67	26	64	0.96	0.70
910	5	36	910	0	790	0.87	0.87
91	5	36	91	0	75	0.83	0.83
880	5	24	816	7	710	0.87	0.81
1300	5	24	1240	5	1060	0.86	0.82
			49.8*		40.2	0.81	

* 7.1 mg. or 49.2 micromoles of isolated triacetic acid.

decarboxylated, while triacetic lactone does not give the direct color reaction.

In Table I are recorded some typical saponification results with two different concentrations of triacetic ester at 5° and 30°. The terms "total" and "free" diketones are employed, since triacetic ester yields only 80 to 82 per cent as much color as triacetic acid in the direct color reaction, while after acid hydrolysis it yields the same amount of color per mole as the free acid (8). "Free" diketone is therefore the value obtained by the direct reaction, "total" the value obtained after acid hydrolysis. During saponification at 5° the free diketone content rose until it was equal to the original "total" diketone, and simultaneously a β-keto acid was produced which with aniline citrate released CO_2 equivalent to 83 to 87 per cent of

the final diketone content of the solution. Thus there was no loss of diketone, although the low CO_2 equivalent was puzzling until it was found that the isolated triacetic acid also yielded only 81 per cent of the theoretical CO_2 on catalytic decarboxylation. In contrast, at 30° there is approximately a 30 per cent loss of diketone, and the apparent equivalence of the CO_2 released and the diketone content of the solution is evidently due to formation of other β-keto acids under these conditions.

After 36 hours saponification at 5°, 80 per cent of the acid could be extracted with ethyl ether, and 50 per cent of the acid was recovered after evaporation of the solvent and drying.

Properties of Triacetic Acid

Triacetic acid is soluble in water, chloroform, ethyl ether, dioxane, and ethyl acetate, but insoluble in petroleum ether and ligroin. The acid gives a red color with ferric chloride. The acid is best stored at 5° *in vacuo* over H_2SO_4. Aqueous solutions of the acid are quite unstable. In 3 hours at 38°, 0.004 M solutions were 36 per cent decomposed over the pH range of 4 to 8, and even at 5° in 0.02 M solution there was a 10 to 15 per cent decomposition at pH 8.0. Based on diketone analysis and catalytic decarboxylation, this decomposition appears to be principally a spontaneous decarboxylation. Solutions of triacetic acid for metabolic experiments are therefore prepared just prior to use.

In Fig. 1 are recorded the ultraviolet absorption curves of triacetic acid and triacetic lactone. It may be noted that the absorption maximum of the lactone is shifted approximately 5 mμ toward the visible region, and that its absorption coefficient is slightly more than twice that of the free acid.

o-Phenylenediamine Reaction—Triacetic acid reacts directly with *o*-phenylenediamine to produce the typical red color. An initial lag in color development is noted as compared with acetylacetone, and under comparable conditions in the respirometer it is clear that the acid undergoes decarboxylation during the period of color development. The same color value is reached as with acetylacetone.

Decarboxylation of Triacetic Acid—Studies were made of the catalytic decarboxylation of triacetic acid compared with acetoacetic acid. Triacetic acid was decarboxylated by aniline citrate (5), aniline acetate (7), aniline acetate in the presence of sodium cyanide (6), *o*- and p-phenylenediamine citrate (7), and by low concentrations of *o*-phenylenediamine (250 mg. per cent) in dilute H_2SO_4 (pH 1.3 to 1.5). The decomposition of triacetic acid by aniline citrate is considerably slower than that of acetoacetic acid, but the reverse is true with *o*-phenylenediamine in dilute sulfuric acid. With the other amine catalysts the rates were roughly equivalent.

After completion of the triacetic acid decarboxylation by aniline citrate, only 81 to 85 per cent of the theoretical CO_2 had been released, and 86 to 89 per cent by o-phenylenediamine citrate compared to a 90 to 95 per cent yield of CO_2 from acetoacetic acid. After completion of the decomposition of triacetic acid with aniline citrate, 90 to 95 per cent of "free" diketone was present, indicating that formation of triacetic lactone was not a significant side reaction during the decarboxylation.

Copper sulfate or aluminum chloride, which catalytically remove one carboxyl from dicarboxylic β-keto acids such as oxalacetic acid or acetone-
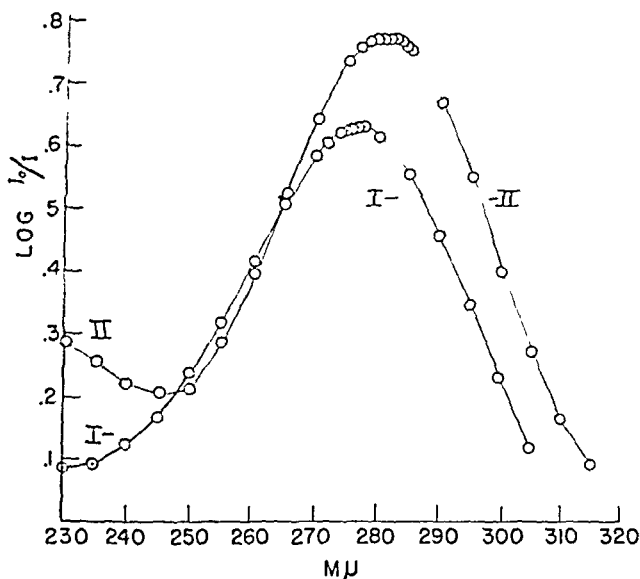
FIG. 1. Ultraviolet absorption spectra of triacetic acid (2×10^{-4} M, Curve I) and triacetic lactone (1×10^{-4} M, Curve II) in water. Beckman spectrophotometer; 1 cm. cells.

dicarboxylic acid (13), does not decarboxylate triacetic acid. On the other hand, triacetic acid is decarboxylated by aniline acetate in the presence of sodium cyanide; under these conditions oxalacetic acid is not attacked (6).

Triacetic Lactone—In contrast to the free acid, triacetic lactone is remarkably stable. A 0.04 M solution of the lactone was not affected by 1 M alkali at 30° for 16 hours, nor by 0.2 M alkali at 60° for 1 hour. Solutions so treated gave no direct color reaction with o-phenylenediamine and were not decarboxylated with aniline citrate, but gave quantitative diketone (acetylacetone) recovery after hydrolysis with sulfuric acid. In this connection the analyses of all the salts of triacetic lactone prepared by Collie (9) correspond to those of the enol lactone and not to those of the

free acid.   Our studies also indicate that the lactone and free acid are not readily interconvertible nor in an equilibrium state in solution.

SUMMARY

Triacetic acid (β,δ-diketohexanoic acid) was synthesized through a series of intermediates of which triacetic lactone (9) and copper ethyl triacetate (4) have been previously described.   Triacetic acid is a light yellow, waxy solid melting at 29–31°, soluble in water and several organic solvents, and unstable in aqueous solution.   Since triacetic acid is a β-keto acid, it is decarboxylated by aromatic amines, and because of its β-diketone group reacts with o-phenylenediamine to produce a red color which is used for quantitative estimation of the acid and its derivatives. Other properties of triacetic acid, triacetic ester, and the lactone are recorded.

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COLORIMETRIC DETERMINATION OF ACETYLACETONE AND RELATED β-DIKETONES*

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There is a lack both of the intermediates often proposed in fatty acid oxidation and synthesis and of methods for their analysis. In the preceding paper (1) the synthesis of triacetic acid (β, δ-diketohexanoic acid) was reported; in this paper is described the colorimetric determination of this compound and related β-diketones. The method is sensitive, quite specific, and is free from interference by acetoacetic acid and acetone. The color reaction involves a condensation of the diketone with o-phenylenediamine in acid solution to produce a reddish purple color, a reaction previously described (2–4). In the case of acetylacetone, which has served as a convenient standard, the reaction may be written as in the accompanying scheme.

2,4-Dimethyl(1,5)benzo-
diazapine hydrochloride
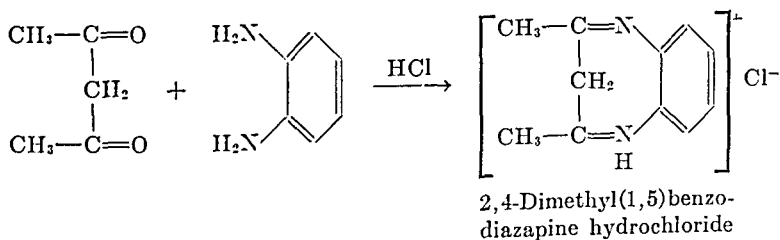
In the reaction with triacetic acid, the acid appears to be decarboxylated to acetylacetone during the period of color development (1). In the case of ethyl triacetate the ester itself probably reacts with the o-phenylene-diamine or at least is only partially hydrolyzed during color development, since the color yield per mole of ester is less than with acetylacetone or the free acid. Triacetic lactone

$$\text{CH}_3\text{—C}\overset{\displaystyle\overset{\text{O}}{\overbrace{}}}{=}\text{CH—CO—CH}_2\text{—CO}$$
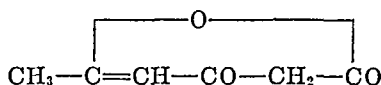
which lacks the β-diketone linkage does not react directly with the reagent at room temperature, but can be converted to acetylacetone by hot acid hydrolysis. A number of qualitative tests for the identification of the purple pigment 2,4-dimethyl(1,5)benzodiazapine are reported, and the extent of interference of various substances determined. The recovery of acetylacetone or triacetic lactone added to various biological systems has been tested. From 2 to 8 micromoles of diketone are recovered to the extent of 95 to 100 per cent.

EXPERIMENTAL

Chemicals and Reagents—Sodium pyruvate was prepared by the method of Robertson (5), and oxalacetic acid by the procedure given by Cohen (6). Triacetic lactone was prepared from dehydroacetic acid by the method of Collie (7). The identification and analysis of this compound are given in the preceding paper (1). The usual grades of ethyl acetoacetate had to be redistilled twice *in vacuo* to remove an impurity that forms a reddish purple color with *o*-phenylenediamine.

1. *Stock acetylacetone.* 1.00 gm. of acetylacetone (freshly distilled *in ̃cuo*) is dissolved in 500 ml. of 0.1 M potassium phosphate buffer of pH ̃, and diluted to 1000 ml. This solution keeps for at least 1 month at 5° the dark.

2. *Phosphate-sulfuric acid reagent.* Dissolve 43.5 gm. of K_2HPO_4 and 0 ml. of 5 N phosphoric acid in 500 ml. of 2.4 N sulfuric acid and dilute o 1000 ml.

3. *o-Phenylenediamine reagent.* Dissolve 100 mg. of *o*-phenylenediamine in 25 ml. of phosphate-sulfuric acid reagent. This reagent should be prepared just prior to use.

Method A (Direct or "Free" Diketone)—This method is used to estimate acetylacetone, triacetic acid, or ethyl triacetate.

To 12 ml. of test solution containing from 2 to 8 micromoles of diketone add 2 ml. of *o*-phenylenediamine reagent, mix, and allow to stand at room temperature for 30 minutes. Compare the color against a reagent blank containing 12 ml. of water and 2 ml. of *o*-phenylenediamine reagent at 500 mμ in a photoelectric colorimeter.

If the test solution is colored or turbid, a blank should be run containing the same amount of test solution and 2 ml. of phosphate-sulfuric acid reagent, and its color value (read against water) subtracted from that of the test solution with *o*-phenylenediamine.

Method B ("Total" Diketone, Triacetic Lactone)—This method is used

principally for the analysis of triacetic lactone but is also applicable to ethyl triacetate. It involves a conversion of either compound to acetylacetone; hence in the determination of either compound a standard acetylacetone curve may be used. In the *direct* method for triacetic ester a factor must be employed. Method B may be used to determine "total" diketone when triacetic lactone is present with "free" diketones. The difference obtained in Methods A and B can be used as a measure of triacetic lactone.

To the distillation flask of a unit such as that described by Stotz (8) are added 13 ml. of test solution containing from 2 to 8 micromoles of diketone, 1 ml. of concentrated sulfuric acid, and a quartz pebble (a few crystals of cholesterol are most useful to prevent foaming during the boiling of filtrates of biological material). The flask is placed in a small heated sand bath to provide a slow, steady boiling so that 10 to 11 ml. of distillate are collected during the course of 15 to 20 minutes. A 25 ml. glass-stoppered cylinder immersed in an ice bath serves as a convenient receiving vessel. At the end of the distillation, the contents of the graduate are diluted to 12 ml. 2.0 ml. of *o*-phenylenediamine reagent are added, the solution mixed, and the color allowed to develop for 30 minutes as in the *direct* method.

Qualitative Tests for Dimethylbenzodiazapine—The following qualitative tests for the typical purple pigment may be found convenient to identify the reaction. In 10 ml. of the colored reaction mixture obtained in the quantitative determination, (1) the color is discharged immediately by the addition of 2 ml. of 10 per cent NaOH, and in 20 to 30 minutes by 40 per cent formaldehyde, 2 M hydroxylamine hydrochloride, or 0.5 M sodium bisulfite, (2) the addition of acid to approximately pH 2.0 restores the color of the solution which has been made alkaline, (3) the leuco pigment is extracted from alkaline solution with chloroform, and the purple color reappears when glacial acetic acid is added, and (4) the purple pigment is quantitatively precipitated with 0.3 ml. of 10 per cent sodium tungstate at pH 1.3 to 2.0. (An orange precipitate separates with the crotonaldehyde-*o*-phenylenediamine compound, and a white precipitate with the diacetyl-*o*-phenylenediamine compound.)

Diketone Color Values—In the *direct* analytical method for diketones a straight line relation was found between color density and concentration of the diketone. This is recorded in Table I, along with the relative color values obtained with the other diketones in the direct reaction. It is evident that triacetic acid yields the same color per mole as acetylacetone, but ethyl triacetate has a lower color yield. Nevertheless a straight line relationship of density to concentration is still obtained with the ester.

Effect of pH and o-Phenylenediamine Concentration—The effects of changes in the pH and *o*-phenylenediamine concentration on the color reaction with acetylacetone are illustrated in Table II. Acetylacetone (8

micromoles) samples were treated with 2 ml. of o-phenylenediamine of various acid strengths and concentrations of diamine. The color density

TABLE I

Relative Color Value Obtained in o-Phenylenediamine Reaction by Different Diketones

Color reaction made according to Method A. Two different preparations of triacetic acid gave identical values. The amount of triacetic ester added was measured from the "total" diketone content according to Method B.

| Amount added | Color value found | | | | | | Triacetic lactone |
| | Acetylacetone | | Triacetic acid | | Ethyl triacetate | | |
micromoles	micromoles*	ratio†	micromoles	ratio	micromoles	ratio	
2.0	2.01	1.01	2.00	1.00	1.66	0.83	0
4.0	4.00	1.00	4.04	1.01	3.22	0.81	0
6.0	6.00	1.00	6.10	1.02	4.90	0.82	0
8.0	7.95	0.99	8.00	1.00	6.47	0.81	0

    * As micromoles of acetylacetone determined from the standard curve.

    † Calculated as the micromoles found divided by the micromoles of substances added.

TABLE II

Effect of o-Phenylenediamine Concentration and pH on Intensity of Color Reaction

8 micromoles of acetylacetone used in each sample.

pH	o-Phenylenediamine	Minimum time of color development	$K \times 10^{-2}$*
	mg.	min.	
1.3	2	60	7.01
1.3	4	20	7.90
1.3	8	20	8.35
0.3	4	10	6.50
1.2	4	20	7.80
2.1	4	60	7.70
3.1	4	90	6.90
0.3	8	10	7.60
1.2	8	20	8.30
2.1	8	30	8.30
3.0	8	60	7.70

    * $K = (2 - \log G)/C$, where G = the galvanometer reading at 500 mμ when the control tube is set at 100, and C = the molar concentration of acetylacetone.

was estimated at 10 minute intervals against the appropriate o-phenylenediamine blank.

With either 4 or 8 mg. of o-phenylenediamine the color density was essen-

tially constant between 1.3 and 2.0 but was decreased when the pH of the solution was decreased to 0.3 or increased to 3.0. Thus the pH and diamine concentration must be controlled, although straight line relations were obtained between color density and diketone concentration except with the smallest amount of o-phenylenediamine. The conditions chosen and described under Method A permit the greatest variation in pH and time of color development without affecting the color value.

Hydrolysis and Recovery of Triacetic Lactone—The strength of acid and time for distillation recommended in Method B are critical for successful recovery of triacetic lactone. Only 15 to 20 per cent of the lactone was

TABLE III

Recovery of Triacetic Lactone from Pure Solution

Compound added		Compound recovered			
Lactone	Acetylacetone	Lactone*		Acetylacetone†	
micromoles	micromoles	micromoles	per cent	micromoles	per cent
2.00		1.97	98		
4.00		3.90	98		
8.00		7.70	96		
4.00	2.00	3.74‡	94	1.98	99
2.00	5.00	1.84‡	92	4.95	99
2.00	2.00	1.80‡	90	2.00	100

* Determined by acid distillation (Method B).

† Determined by Method A.

‡ Calculated from the difference in color density of equal aliquots determined by Methods A and B.

hydrolyzed when 1 ml. of 10 per cent metaphosphoric acid was used in place of 1 ml. of sulfuric acid, and 74 to 78 per cent was hydrolyzed when 0.5 ml. of sulfuric acid was employed. About 74 to 88 per cent recovery was obtained if the time of distillation was lowered to 8 to 10 minutes; hence the fairly slow steady distillation recommended is essential. Simultaneous separation of the acetylacetone formed upon hydrolysis of the lactone seemed essential, since separation of the hydrolysis and distillation steps did not give satisfactory yields.

With the conditions recommended, 96 to 98 per cent recoveries of triacetic lactone have been obtained. Typical results are recorded in Table III. In practice, duplicate samples have checked within 2 per cent. At least some of the lowered yield in the determination of triacetic lactone is probably due to destruction of the acetylacetone liberated in the strong acid solution, since a 2 to 5 per cent loss was observed if acetylacetone was substituted for the lactone. Table III also demonstrates that triacetic lactone can be estimated in the presence of acetylacetone, and here the apparently

lowered yield of triacetic lactone is evident, owing for the most part to acid destruction of the free acetylacetone present.

The distillation procedure may be convenient for analysis of acetylacetone or triacetic acid if non-volatile interfering substances are known to be present.

Specificity and Interfering Compounds—No color was formed with o-phenylenediamine under the conditions of Method A with 100 micromoles of the following compounds: diacetyl, acetoin, acetone, acetaldehyde, succinic acid, fumaric acid, acetoacetic acid, oxalacetic acid, levulinic acid, citric acid, ethyl acetoacetate, ethyl levulinate, or diethyl acetonedicarboxylate. Since these compounds were tested in a concentration over 10 times that of the acetylacetone, the color reaction appears to be specific. On the other hand the color reaction is inhibited by certain substances. Tungstic acid precipitates the pigment at pH 1.3 to 2.0, oxidizing agents such as dichromate or peroxide oxidize the o-phenylenediamine, and bisulfite, hydroxylamine, or semicarbazide inhibits color formation. An inhibition of the color reaction of 10 to 15 per cent was observed in the presence of 100 micromoles of pyruvic acid (used as the purified sodium salt) and a complete inhibition occurred with 100 micromoles of diacetyl or formaldehyde. Reduction of pyruvate to 50 micromoles and diacetyl to 10 micromoles results in a 93 to 95 per cent color yield with acetylacetone. 100 micromoles of c.p. ascorbic acid did not cause an inhibition of color development, but some samples of U. S. P. ascorbic acid at a level of 50 micromoles caused a 10 to 15 per cent inhibition. Glucose in 10 per cent solution or 0.4 M urea did not interfere in the direct determination of acetylacetone.

Crotonaldehyde in amounts of from 10 to 50 micromoles gives an instantaneous yellow to orange color upon addition of o-phenylenediamine. Thus when 50 micromoles of crotonaldehyde are present, 2 micromoles of acetylacetone appear to give a 135 per cent yield, 4 micromoles a 115 per cent yield, and 8 micromoles a 105 per cent yield. The crotonaldehyde interference can be eliminated by the addition of 2 ml. of 0.05 M sodium bisulfite to 10 ml. of the solution after the usual 20 to 30 minutes of color development when the color is read after 15 to 20 minutes against a reagent blank containing bisulfite. Bisulfite causes a decrease of 40 per cent in the color owing to acetylacetone but a straight line relationship between color density and acetylacetone concentration is still observed.

With certain exceptions the effects of the interfering substances are the same in the triacetic lactone distillation procedure as in the direct acetylacetone method. High concentrations of glucose apparently yield volatile split-products during the hot acid distillation which give orange to red colors with o-phenylenediamine. Interference is not serious, however, until 1 per cent glucose is present. Low recoveries (67 to 70 per cent) of triacetic lactone were observed in the presence of 0.4 M urea, a concentra-

tion which may be found in urine. The same effect was observed in the distillation of acetylacetone from sulfuric acid solution but not from metaphosphoric acid. Since urea or its distillation products do not interfere with the direct color reaction, it appears that urea condenses with acetylacetone under the hot acid conditions necessary for the hydrolysis of triacetic lactone.

Although Elson and Morgan (9) reported a method for the estimation of glucosamine based on its condensation with acetylacetone in hot acid solu-

TABLE IV

Recovery of Acetylacetone and Triacetic Lactone Added to Biological Materials

Biological material	Triacetic lactone			Acetylacetone		
	Added	Recovered		Added	Recovered	
	micro-moles	*micro-moles*	*per cent*	*micro-moles*	*micro-moles*	*per cent*
10% kidney mince	3.20	3.14	98			
	6.40	6.10	95			
				4.24	4.10	97
				2.14	2.02	94
20% glucose "residual harvest medium"				2.22	2.15	97
				4.22	4.06	96
				8.10	7.90	98
8% *Fusarium lycopersici* suspension	2.50	2.40	96			
				2.50	2.36	94
Extract of *Fusarium lycopersici*	2.00	1.90	95			
	6.40	6.25	98			
Suspension of *Escherichia coli*				4.00	4.00	100
0.2% peptone "residual harvest medium"	4.00	3.75	94			
Urine, direct procedure				5.26	5.35	102
				9.26	9.26	100
" distillation procedure without sulfuric acid				8.00	7.85	98

tion to form pyrrole derivatives, 50 micromoles of glucosamine did not interfere in the determination of triacetic lactone.

Recovery of Diketones from Biological Systems—In Table IV are presented results in which different amounts of acetylacetone or triacetic lactone were added to various biological systems. Metaphosphoric acid filtrates were preferred for general use, with 2 per cent acid as a final concentration. In the acetylacetone determination, if the metaphosphoric acid filtrate was obviously turbid or colored, a blank in addition to the o-phenylenediamine control was run. In this control, phosphate-sulfuric acid reagent was substituted for o-phenylenediamine as recommended under Method A. An internal blank may be found convenient in some cases by discharging the color with 2 ml. of 40 per cent formaldehyde and estimating the residual color density.

In none of the biological materials analyzed was there any evidence for a measurable amount of acetylacetone, triacetic acid, or triacetic lactone occurring naturally. The systems used were representative of the types in which triacetic acid metabolism might be studied, and provided for the testing of possible interfering materials. The biological systems tested included (a) 10 per cent kidney mince, (b) a 10 per cent glucose medium (10) from which a high fat mycelial mat of *Fusarium lycopersici* (11) had been harvested (called "residual harvest medium" in Table IV), (c) an 8 per cent cell suspension of *F. lycopersici*, (d) an extract of *F. lycopersici* prepared by grinding 5 gm. of lyophilized mold with powdered glass (12) and suspending in 100 ml. of water, (e) a suspension of *Escherichia coli* (20 mg. of dry weight per ml.), (f) a peptone medium (13) from which *Escherichia coli* was collected after 48 hours of aerobic growth at 37°, and (g) urine. Satisfactory recoveries of both acetylacetone and triacetic lactone were found with these biological materials.

SUMMARY

Methods are presented for the colorimetric determination of from 2 to 8 micromoles of the β-diketones: acetylacetone, triacetic acid, ethyl triacetate, and triacetic lactone. The lactone is first converted to acetylacetone by acid hydrolysis and separated by distillation. The color reaction depends on a condensation of the diketones in acid solution with o-phenylenediamine to form the reddish purple dimethylbenzodiazapine. The specificity of the reaction has been studied and the limiting concentrations of interfering substances determined. Recoveries of acetylacetone and triacetic lactone added to a variety of biological systems are given.

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THE METABOLISM IN VITRO OF TRIACETIC ACID AND RELATED DIKETONES*

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Polyketonic acids have been postulated as intermediates in theories of fatty acid oxidation (1–3) and synthesis (4), but direct evidence has been generally lacking. The α,γ-diketo acid, acetopyruvic acid, is apparently rapidly metabolized by animal tissues (5, 6), and an enzyme which produces pyruvic acid from this compound has been partially purified by Meister and Greenstein (7). However, Lehninger (8) found that α,γ-diketo-octanoic acid was only slowly metabolized by broken cell preparations of liver, in contrast to octanoic acid itself, and the study of Weinhouse and coworkers (9) with carboxyl-labeled octanoic acid leave little place for α,γ oxidation as a mechanism for the oxidation of fatty acids with an even number of carbon atoms.

β oxidation is of course the more generally accepted basis for fatty acid oxidation. It may be pointed out, however, that none of the existing theories of fatty acid oxidation is based on evidence which precludes multiple oxidation of the fatty acid before splitting, but that the conflicting theories differ mainly in the suggested mode of cleavage of the oxidized fatty acids or in subsequent reactions. Polyketonic acids, resulting from successive condensations of acetic acid molecules, might also be postulated as intermediates in fat synthesis.

In spite of the probable importance of multiple alternate β-oxidized fatty acids in fat metabolism, representatives of this class of compounds have not been available for testing. In the preceding papers (10, 11) the preparation and determination of triacetic acid (β,δ-diketohexanoic acid) have been described. In this report it will be shown that triacetic acid is readily metabolized by liver tissue *in vitro*, with the formation of acetoacetic acid and acetic acid, a finding which necessitates the consideration of such "multiple alternate" oxidized fatty acids in theories of fat metabolism.

During the course of our investigations, Breusch and Ulusoy (12) reported that the δ-lactone of triacetic acid (referred to by these investigators

as "triacetic acid") was metabolized by liver at such a rate as to make it possible that this substance is an intermediate in fatty acid oxidation. These investigators found that approximately 1.3 moles of acetoacetic acid were formed from 1 mole of the lactone, a finding which might support β splitting and recondensation of 2-carbon fragments as a mechanism of hexanoic acid oxidation. Free acetic acid could not be found as a product of the reaction.

Analytical Methods—Since the β-diketones were found to interfere in the several methods tested for the determination of acetone, it was first necessary to develop a procedure for the preliminary removal of these compounds. This was accomplished by precipitation of the colored acetylacetone-*o*-phenylenediamine complex with tungstic acid. Acetone was then determined by a modification of the vanillin method (13) which seemed to offer the greatest specificity.

Removal of Acetylacetone Prior to Acetone Analysis—Metaphosphoric acid filtrates containing both acetoacetic acid and triacetic acid are distilled, and the *o*-phenylenediamine color reaction for acetylacetone is carried out on the filtrate as described previously (11). Acetone present in the filtrate does not react with the *o*-phenylenediamine reagent. For removal of the colored complex, 0.3 ml. of 10 per cent sodium tungstate is then added, and the solution allowed to stand overnight at 5°. After the centrifuged precipitate is discarded, 10 ml. of the colorless solution are distilled into 1 ml. of water contained in a 25 ml. glass-stoppered cylinder placed in an ice bath. Approximately 8 ml. of distillate are collected, and the volume adjusted to 10 ml. A 1 to 2 ml. portion of this filtrate is used for the vanillin reaction to determine acetone, as described below.

In practice both diketones and acetone can be determined on the same tissue filtrate by measuring the intensity of the *o*-phenylenediamine color reaction in the distillate, followed by removal of the colored complex with tungstate and estimation of acetone.

Vanillin Reaction—The following reagents were employed: (1) potassium hydroxide, 100 gm. dissolved in 60 ml. of distilled water; (2) 10 per cent vanillin (Eastman, practical grade) in acetone-free methyl alcohol, prepared fresh for use; (3) acetone stock standard, 0.01 M acetone, standardized by the iodoform reaction; this solution keeps at least for a month at 5°; (4) acetone working standard, 0.1 to 0.5 micromole of acetone per ml., freshly prepared.

Procedure—To 2 ml. of acetone solution (0.2 to 1.0 micromole of acetone) contained in a test-tube are added 2 ml. of potassium hydroxide, with cooling in an ice bath. After the tube is removed from the ice bath, 1 ml. of vanillin reagent is added and the tube shaken for thorough mixing. The gelatinous precipitate formed dissolves on subsequent heating. A clean

rubber stopper is inserted, and the tube heated at 65° for 20 minutes. After cooling, 10 ml. of water are added, the tube contents mixed, and the color allowed to develop for 15 minutes. The color may be estimated by a Lumetron colorimeter with the 515 mμ filter or by the Klett-Summerson colorimeter with the green filter. A vanillin "control" without acetone is employed as well as "standard" tubes containing from 0.2 to 1.0 micromole of acetone.

Under a given set of conditions a linear relation between acetone concentration and color density is obtained, but standard curves may vary as much as 10 per cent; hence standards are always employed with a set of unknowns. Methyl ketones other than acetone do not give the vanillin reaction, but acetylacetone gives the same color per mole. Acetylacetone is apparently converted to acetone by alkali, a reaction previously noted (14). After removal of the colored acetylacetone-o-phenylenediamine complex with tungstic acid, as previously described, the distillates from such treatments still yield a slight blank in the vanillin reaction, equivalent to 0.0 to 0.03 micromole of acetone. This blank was regularly deducted in experiments involving both diketones and acetone, but always represented less than 5 per cent of the acetone present.

In Table I are presented the results of acetone recovery, alone and in the presence of acetylacetone.

Triacetic acid, ester, and lactone were determined by the methods previously described (11), total β-keto acids by the aniline citrate decarboxylation method (15), and β-hydroxybutyric acid by a modification of the Barnes and Wick method (16).

Utilization of Triacetic Acid, Ester, and Lactone by Tissue Homogenates

Rats were fasted overnight, sacrificed, and tissue homogenates prepared by grinding 1 part of tissue with 3 parts of isotonic saline at 5° for 5 minutes in a mechanical glass homogenizer (17). The usual reaction mixture was composed of 0.5 ml. of 0.01 M substrate, 0.2 ml. of 0.1 M sodium phosphate buffer (pH 7.4), 0.4 to 0.8 ml. of tissue homogenate, and distilled water to make a volume of 1.5 ml. Such mixtures were incubated in stoppered tubes at 30° with shaking. At zero time and at various intervals thereafter, 6 ml. of 5 per cent metaphosphoric acid were added to stop the reaction, and the mixture diluted to 15 ml. Analyses were carried out on the supernatant fluids. Since a linear relationship was found between substrate disappearance and time of the reaction, it was customary to determine the substrate concentration at zero time and at two subsequent time intervals. The results are reported as micromoles of triacetic compound which disappeared per ml. of tissue preparation per hour. Several representative experiments are reported in Table II.

TABLE I

Recovery of Acetone Alone and in Presence of Acetylacetone

Acetylacetone added	Acetone	
	Added	Found
micromole	*micromole*	*micromole*
0	0.56	0.56
0	0.42	0.42
0	0.28	0.30
0.14	0.00	0.01
0.14	0.56	0.54
0.14	0.42	0.42
0.14	0.28	0.30
0.35	0.00	0.02
0.35	0.56	0.57
0.35	0.42	0.44
0.35	0.28	0.26

TABLE II

Metabolism of Triacetic Acid, Ester, and Lactone by Tissue Homogenates

Tissue	Time of incubation	Triacetic substrate	Activity*	Ratio†
	min.			
Rat liver	40	Acid	12.1	0.90
" "	40	Ester	10.8	0.95
" "	80	Lactone	1.3	0.90
" "	45	Acid	11.6	1.00
" "	45	Ester	9.8	1.05
" "	90	Lactone	0.9	0.90
" "	45	Acetylacetone	0	
" "	45	Acid		1.00
" "	45	Ester		0.90
" "	90	Lactone	1.3	0.95
" "	30	Acid	9.0	1.00
" "	45	Acetoacetate	0	
" kidney	30	Acid	5.9	1.05
Beef liver	60	"	3.6	
" "	60	Lactone	0	
Rabbit liver	40	Acid	5.9	
" "	120	Lactone	0.8	
" kidney	40	Acid	1.1	
" "	120	Lactone	0.4	

* Micromoles of substrate which disappeared per ml. of homogenate employed per hour.

† Micromoles of acetoacetate formed per micromole of triacetic substrate which disappeared.

It may be seen that triacetic acid and ester were metabolized by rat liver homogenate at approximately the same rate, while triacetic lactone disappeared at only about one-eighth this rate. The rate of disappearance of the substrates was not affected by the absence of oxygen, nor by the addition of adenosine triphosphate, Mg^{++}, or diphosphopyridine nucleotide. In connection with these findings, the relative rates of disappearance of triacetic acid and hexanoic acid have been compared. Homogenates with appropriate additions according to Lehninger (18) caused a triacetic acid disappearance about 1.5 times as rapid as that of hexanoic acid.

Triacetic acid was not metabolized by rat heart, skeletal muscle, spleen, or pancreas.

Formation of Acetoacetic and Acetic Acids from Triacetic Acid in Rat Liver Homogenates—A determination of the acetoacetic acid formed during the metabolism of triacetic compounds in liver was also made on the same reaction mixture. These results are also recorded in Table II.

1 mole of acetoacetic acid was regularly formed from 1 mole of triacetic acid, ester, or lactone. This was true whether 50 per cent or 90 per cent of the triacetic acid had disappeared. Added acetoacetic acid or acetylacetone did not disappear from the reaction mixture, nor was any β-hydroxybutyric acid formed from triacetic acid. The same ratio of acetoacetic acid formed to the triacetic compound which disappeared was observed in the presence of adenosine triphosphate, Mg^{++}, and cytochrome c, conditions which provided for an active oxidation of hexanoic acid with acetoacetate formation.

There seemed to be no alternative than acetic acid for the other product of triacetic acid breakdown, according to the equation, $CH_3COCH_2COCH_2COOH + H_2O \rightarrow CH_3COCH_2COOH + CH_3COOH$.

The formation of acetic acid was observed in experiments conducted on a scale larger than usual, as follows: 4.0 ml. of 0.1 M phosphate buffer (pH 7.4), 16 ml. of 25 per cent rat liver homogenate, and 10 ml. of 0.02 M triacetic acid were incubated at 30° for 90 minutes. Simultaneous controls measured the formation of volatile acid in the absence of triacetic acid, and the recovery of added acetic acid. Metaphosphoric acid filtrates were prepared. The volatile fatty acids were distilled after addition of 25 gm. of magnesium sulfate (19) to 100 ml. of metaphosphoric acid filtrate, and the distillation continued until crystallization of the residue in the distilling flask was observed. The distillate was boiled for 3 to 5 minutes with almost complete removal of acetylacetone formed from the residual triacetic acid. The solution was then titrated with 0.01 M alkali. Neither acetoacetic acid nor acetylacetone interfered with the determination as described at the concentrations which occurred in these experiments. Chloride and sulfate tests on the distillates were negative.

In a typical experiment 184 micromoles of triacetic acid disappeared in 90 minutes; 164 micromoles of acetoacetic acid and 169 micromoles of volatile fatty acid were recovered. The yield of volatile fatty acid was corrected for an 85 per cent recovery of acetic acid from a liver homogenate and for "endogenous" fatty acids equivalent to about 8 per cent of the acid found in the experimental sample. Similar recoveries of acetic acid have been reported by other workers under similar analytical conditions (20, 21).

For the identification of the volatile fatty acid as acetic acid, the solution after alkali titration was evaporated to a small volume, acidified, and redistilled. The Duclaux number of the volatile fatty acid was determined parallel with samples prepared in the same way after addition of equivalent quantities of acetic acid to liver homogenates. The data in Table III show that the Duclaux number of the unknown acid is very similar if not identical

TABLE III

Duclaux Constants of Acid Produced from Triacetic Acid in Liver Homogenates

Acid	Per cent distilled			Total acid distilled
	Fraction 1	Fraction 2	Fraction 3	
				per cent
Unknown	22.4	25.3	34.9	82
Acetic acid added to homogenate..........	21.6	24.6	33.2	79
Acetic acid	20.8	24.6	33.2	79
Propionic acid...........................	37.8	33.2	26.5	97.5

with that of acetic acid, and readily distinguished, for example, from that of propionic acid. A positive lanthanum nitrate test (22) for acetic acid was also obtained on the volatile fatty acid fraction.

Triacetic Acid Splitting with Purified Liver Enzyme—By means of fractional alcohol precipitation and selective heat denaturation, a 100-fold purified enzyme was prepared from beef liver which catalyzed the conversion of 1 mole of triacetic acid to 2 moles of acid.[1] Acid production was followed manometrically in the Warburg apparatus with bicarbonate buffer and a gas atmosphere of 95 per cent N_2-5 per cent CO_2 at 30°, pH 7.4. The results are recorded in Table IV.

For each mole of triacetic acid which disappeared, 1 mole of acetoacetic acid and an extra mole of another acid were formed. The acetoacetic acid was determined both by the colorimetric method and the manometric decarboxylation procedure. Acetic acid was indicated here as the other

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[1] The purification and properties of this enzyme will be the subject of a later communication.

acid formed by a positive lanthanum nitrate test on the volatile fatty acid fraction from the reaction mixture.

The purified enzyme did not attack acetylacetone, triacetic ester, triacetic lactone, β-ketohexanoic acid, or acetoacetic acid.

Ethyl Triacetate Metabolism—In tracing the mechanism involved in the disappearance of triacetic ester from rat liver homogenates, the analytical problem was to determine the triacetic ester, free triacetic acid, and acetoacetic acid in the same reaction mixture.   The total β-diketones (ester and acid) were estimated by the o-phenylenediamine procedure, and acetoacetic acid by the vanillin reaction after β-diketone removal.   Total β-keto acids (acetoacetic plus triacetic) were determined by the manometric decarboxylation procedure, and from these values the triacetic ester could be calculated.

TABLE IV

Splitting of Triacetic Acid by Purified Liver Enzyme

10 micromoles of triacetic acid in 3.3 ml. volume in Warburg flasks.   Bicarbonate buffer, pH 7.4; temperature 30°.

Enzyme	Time of incubation	Triacetic acid disappeared (chemical analysis)	Acetoacetic acid formed	Extra acid formed (manometric determination)
ml.	*min.*	*micromoles*	*micromoles*	*micromoles*
0.2	40	3.6	4.0	3.4
0.4	40	8.0	8.0	7.0
0.6	40	9.2	9.3	8.9
0.5	30	9.2	9.4	8.4
0.5	30	8.1	7.9	7.2

Experiments were conducted with liver homogenates, purified liver esterase,[2] and purified triacetic acid enzyme.   The results recorded in Table V make it clear that the utilization of triacetic ester involves a preliminary hydrolysis of the ester by liver esterase, with subsequent splitting of the liberated triacetic acid.   In crude liver homogenates the accumulation of the free triacetic acid can be demonstrated with malonate, since this substance has been found to inhibit greatly the splitting of triacetic acid in such homogenates.

Triacetic Lactone Metabolism—As recorded previously, triacetic lactone is metabolized more slowly than the free acid by rat liver homogenate, with the formation of 1 mole of acetoacetic acid from 1 mole of triacetic acid. The latter point is in disagreement with the results of Breusch and Ulusoy (12) who found approximately 1.3 moles of acetoacetic acid formed from

[2] Dr. A. L. Dounce and coworkers in this laboratory have prepared a highly purified liver esterase.

1 mole of triacetic lactone. They concluded that this was sufficiently close to a theoretical 1.5 moles to support a β oxidation-recondensation type of metabolism for this compound. The reason for the difference in results obtained is not clear, but probably rests in the specificity and accuracy of the analytical methods employed.

In any event it is felt that the free acid is the more likely diketone metabolite if such compounds occur in fat metabolism, and this is supported by its much greater rate of metabolism. Furthermore the lactone of triacetic acid cannot be considered as being in simple chemical equilibrium with the free acid. This was pointed out in a previous paper (10) and is further emphasized by the finding that the purified triacetic acid enzyme does not affect the lactone. It seems probable that the formation of acetoacetic

TABLE V

Metabolism of Ethyl Triacetate by Liver Enzymes

Incubation at 3 3 ml.; bicarbonate buffer, pH 7.4; temperature 30°; malonate 0.01 M.

Enzyme	Malonate	Time	Conversion of ester to	
			Triacetic acid	Acetoacetic acid
		min	*per cent*	*per cent*
Liver homogenate	+	20	87	0
" "	—	20	58	37
" "	+	60	58	25
" "	—	60	0	90
Triacetic acid enzyme	—	55	0	0
Esterase	—	55	86	
" + triacetic acid enzyme	—	55		92

acid from triacetic lactone involves a preliminary formation of the free triacetic acid, but this point has not been thoroughly investigated. In this connection Breusch and Ulusoy found that the enzyme in liver homogenate which acts on the lactone was destroyed by heating for 5 minutes at 55°, a condition which we have found does not destroy the triacetic acid enzyme.

DISCUSSION

The data presented have demonstrated the cleavage of triacetic acid into acetoacetic and acetic acids by liver homogenates and by an enzyme isolated from liver, according to the equation $CH_3COCH_2COCH_2COOH \rightarrow CH_3COCH_2COOH + CH_3COOH$. This is the first demonstration of the biological activity of an open chain polyketonic acid which could be formed by "multiple alternate" β oxidation of a fatty acid. In conjunction with the fact that this postulated intermediate is metabolized at least as fast as the

corresponding fatty acid, it would appear necessary to accommodate this type of compound in theories of fat metabolism.

In postulating triacetic acid as an intermediate of hexanoic acid oxidation, the acetoacetate yield from lower fatty acids demands consideration, since triacetic acid yields only 1 mole of acetoacetate. Either β or δ cleavage of triacetic acid is indicated with no recondensation of the resulting 2-carbon fragments. At least in the case of octanoic acid, β oxidation and cleavage with recondensation of 2-carbon units are indicated as the principal mode of acetoacetate production (9, 23). In the case of hexanoic acid, however, experiments on acetoacetate yield *in vitro* are somewhat at variance.

Leloir and Muñoz (24) and Jowett and Quastel (2) find the yield of acetoacetate from hexanoic acid greater than from butyric acid. On the other hand, calculations from Cohen's data (25) indicate the production of 1 mole of acetoacetate from hexanoic acid. The isotope experiments of Morehouse and Deuel (26) with hexanoic acid favor δ cleavage of a multiple "alternate oxidized" intermediate. It is of course possible that the mode of oxidation and cleavage of hexanoic and octanoic acids differs, and further experiments on the oxidation of hexanoic acid *in vitro* are in progress in this laboratory.

Successive condensation of acetic acid molecules of 2-carbon residues from carbohydrate catabolism is indicated in the biological synthesis of fatty acids (27). The pyruvate → acetoacetate reaction (28) and the acetate-acetoacetate conversion (21, 29) offer possibilities for the initial stages of fatty acid synthesis. Further stages are obscure, although the isotope experiments of Rittenberg, Schoenheimer, and Evans (30) do not favor butyric and hexanoic acids as intermediates in the synthesis. Further condensation of acetoacetate with acetate would yield triacetic acid, the intermediate under consideration in this paper. Thus polyketonic acids are implicated in fatty acid synthesis as well as catabolism.

SUMMARY

1. A method for the determination of acetoacetic acid in the presence of triacetic acid and related compounds has been described.

2. Triacetic acid is rapidly metabolized by rat liver homogenate and an enzyme isolated from liver, yielding in both cases 1 mole of acetoacetate and 1 of acetate.

3. Triacetic ester is rapidly metabolized by liver through the successive action of an esterase and the triacetic acid enzyme. Triacetic lactone is metabolized by rat liver at about one-eighth the rate of the free acid.

4. The implications of these findings to studies of fat metabolism are discussed.

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STUDIES ON NITROGEN METABOLISM IN TOMATO WITH USE OF ISOTOPICALLY LABELED AMMONIUM SULFATE*

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The stable isotope of nitrogen, N^{15}, has been employed extensively as a tracer in studies of nitrogen transformations in animals and bacteria, and its use has yielded much information difficult or impossible to obtain otherwise. The extension of this method to studies involving the higher plants, however, has been very limited. Hevesy and coworkers (1) studied the absorption of ammonium ions labeled with N^{15} in the sunflower and showed that there was a definite replacement of the nitrogen present in the mature leaf. Vickery and others (2) employed tobacco and buckwheat and found at the end of 72 and 47 hours, respectively, incorporation of N^{15} from $N^{15}H_4Cl$ into the principal nitrogenous fractions of the plants.

The absorption, translocation, and utilization of inorganic nitrogen by plants has been extensively investigated by means of traditional methods. The demonstration in animals of a rapid and continuous interchange between the various forms of nitrogen (3), however, raises serious doubt concerning the interpretation of some of these earlier findings. Studies of the metabolic pathways for the incorporation of inorganic nitrogen into tissue protein and other nitrogenous constituents of higher plants have therefore been begun with the stable isotope of nitrogen as a tracer. This communication presents certain limited observations of nitrogen metabolism in tomato plants.

EXPERIMENTAL

Materials and Methods

Tomato plants (*Lycopersicon esculentum* Miller, var. John Baer) were used throughout this study. Young seedlings, sprouted in soil, were transferred to silica sand in 10 inch varnished clay pots. A complete nutrient solution (4) containing both ammonium and nitrate nitrogen was supplied. The plants were grown under normal greenhouse conditions of light and temperature, the latter ranging from 24–29° during the day and held near 18° during the night.

Experiment I—Seedlings 6 weeks old were employed in this study. 14 days before the administration of the labeled isotope, the pots were thoroughly leached and during the ensuing period supplied a nutrient solution without nitrogen. At the end of this period of deprivation, distinct symptoms of nitrogen deficiency had appeared. At 9.00 a.m. 15 mg. of ammonium nitrogen as $(N^{15}H_4)_2SO_4$ containing 30 atom per cent N^{15} excess were supplied each pot. The drain was closed with a stopper and distilled water added to saturation. The greenhouse temperature was near 27° and transpiration was rapid. 4 hours later the plants were harvested, separated into tops and roots, and the latter thoroughly washed in tap water to remove adhering salts. The plant parts were rapidly heated in a steam-jacketed oven to approximately 65° and then transferred to a forced draft oven maintained at 60° for 8 hours. After an additional 18 hours in an ordinary 60° oven, the tissues were weighed, the adhering sand removed from the roots, and the parts ground in a mortar to pass a 40 mesh sieve. In this manner 10.5 gm. of dry leaf plus stem tissue and 4.1 gm. of dry root tissue were obtained. Nitrogen fractionation was performed upon these dry materials.

Experiment II—This experiment was essentially a repetition of Experiment I, but slightly larger seedlings, about 2 months old, were used. Nitrogen deficiency was induced by omission of nitrogen from the nutrient solution from March 15 to 27, 1946. On the last day 15 mg. of ammonium nitrogen as $(N^{15}H_4)_2SO_4$ containing 30 atom per cent N^{15} excess were supplied at 7.00 a.m. After 12 hours of conditions conducive to a high transpiration rate (temperature, 24–27°, light intensity, 850 to 950 foot candles at the bench surface) the plants were harvested, heated, and dried as before. The tissues were ground in a Wiley mill and stored for analysis.

Nitrogen Fractionation Procedures—The nitrogen fractionation procedures employed were essentially those of Vickery and coworkers (5), and consisted of ethanol extraction in a continuous drip extractor for 12 hours (ethanol-soluble fraction) followed by repeated extraction with small portions of boiling water, centrifugation, and filtration through a small plug of glass wool (water-soluble fraction). The hot water-insoluble nitrogen has been shown to consist almost wholly of proteins (protein fraction). This latter material was then hydrolyzed with 8 N H_2SO_4 under a reflux for 24 hours, the humin filtered off (humin nitrogen), the sulfate ions removed with $Ba(OH)_2$, and the free amino acids precipitated at approximately pH 8 by mercuric acetate followed by the addition of alcohol to a final concentration of 30 per cent. The insoluble material (Neuberg precipitate) was separated from the liquid (Neuberg filtrate) by centrifugation. Mercury was removed from the acidified Neuberg precipitate as the sulfide; the basic amino acids were precipitated from the resulting solution by the addition of phosphotungstic acid (PTA precipitate and PTA filtrate). In Experiment II,

following the precipitation of the amino acids and similar compounds by the Neuberg procedure, various amino acids or amino acid groups were isolated. The methods employed were essentially those described by Schoenheimer, Ratner, and Rittenberg (6).

Measurement of Isotope Concentration—The concentration of N^{15} in the various fractions and compounds was determined by Kjeldahl digestion, distillation of NH_3 from alkaline solution, and subsequent conversion to N_2 by alkaline hypobromite. Measurement of isotope concentration in the N_2 was made with a mass spectrometer of the Nier type; the error of the determinations was less than ±0.05 atom per cent. Isotope concentration is expressed as atom per cent N^{15} excess.

TABLE I

Isotope Concentration of Various Nitrogen Fractions of Tomato Leaf, Stem, and Root Tissue Supplied $(N^{15}H_4)_2SO_4$ for 4 Hours

The results are expressed as atom per cent N^{15} excess

Nitrogen fraction	Isotope concentration	
	Root tissue	Leaf and stem tissue
Total nitrogen............... . . .	2.42	0.33
Water-soluble.........	7.09	0.71
Ammonia.............. . .	12.06	1.15
Amide	2 17	0.10
Remaining water-soluble..... . . .	1 89	0.63
Humin of hydrolysate.	0 45	0 06
Neuberg filtrate........	0 18	0 25
" ppt.	0 27	0 08
PTA filtrate.......... .	0 24	0 08
" ppt.	0 31	0 08

Results

The isotope concentrations of various nitrogenous fractions of the rapidly growing tomato plants from Experiment I are presented in Table I. Examination of the data reveals that during the 4 hour period rapid assimilation of the labeled ammonium ion occurred; the total nitrogen of root and top attained values of 2.42 and 0.33 atom per cent N^{15} excess, respectively. The ammonia fraction of root tissue contained 12.06 atom per cent N^{15} excess; thus, the absorbed ammonia nitrogen represented some 40 per cent of the total ammonia nitrogen present in the roots after 4 hours.

The data obtained in Experiment II are presented in Table II. It will be noted that in these larger, more mature plants the concentration of isotopic nitrogen attained in 12 hours in the roots was considerably less than that attained in the roots of the younger plants (Experiment I) in 4 hours.

However, the 12 hour period permitted more extensive translocation and assimilation of N^{15} in the tops than did the 4 hour treatment. Considerable difficulty was experienced in isolating the amino acids from the small amount of protein from the root tissue. Several additional amino acids were obtained, but not in sufficient yield to permit satisfactory purification. Likewise in the leaf tissue protein hydrolysates, the small quantity of total amino acid nitrogen did not permit a separation of the individual amino

TABLE II

Isotope Concentration of Amino Acids and Amino Acid Fractions of Tomato Leaf, Stem, and Root Tissue Supplied ($N^{15}H_4)_2SO_4$ for 12 Hours

The results are expressed as atom per cent N^{15} excess.

Fraction	Isotope concentration	
	Root tissue	Leaf and stem tissue
Total nitrogen	1.13	1.02
Ethanol-soluble	3.89	1.74
Water-soluble	0.77	0.49
Protein fractions		
Hydrolysate	0.79	0.58
Humin	0.45	0.21
Neuberg ppt	0.56	0.64
" filtrate	0.68	0.25
NH_3 + amide	0.38	0.72
PTA ppt		0.56
" filtrate		0.82
Water-insoluble copper salts		0.71
Methanol-soluble " "		0.84
Arginine		0.77
Glutamic acid	1.15	4.16
Aspartic "	0.75	0.94
Histidine		0.21
Lysine		0.07

acids present in the copper salts fractions. According to Town (7) the principal amino acids found in the various copper salt fractions are as follows: water-insoluble copper salts, leucine, phenylalanine, cystine, methionine, and any tyrosine or aspartic acid not previously removed; methyl alcohol-soluble copper salts, proline, hydroxyproline, isoleucine, and valine.

DISCUSSION

The speed with which rapidly growing seedlings absorb, translocate, and utilize inorganic nitrogen is clearly shown from the data of Experiment I. During a period of 4 hours, the amount of nitrogen taken into and retained by tomato roots was roughly 8 per cent of the total assimilated by the roots

in their entire growth. During the same period the tops accumulated N^{15}-labeled nitrogen equivalent to about 1.1 per cent of their total nitrogen. This rapid absorption was probably accentuated by the nitrogen-deficient state of the plants. Conditions for rapid translocation also existed during this period and likely promoted rapid upward carriage of the absorbed ions.

Subsequent to the addition of N^{15}-enriched ammonia the N^{15} was incorporated into amino acids and tissue proteins. The data do not reveal how much of the N^{15} of the proteins was incorporated by total synthesis of the proteins and how much by exchange of amino acids with the amino acids of preformed proteins. Although the total nitrogen assimilated in the tops was quantitatively greater than in the roots, the percentage of most nitrogenous fractions formed during the period of treatment was greater in the roots, as attested by their higher atom per cent N^{15} excess.

Glutamine and asparagine, produced in quantity by certain plants during their early growth, are depleted as the plants mature. The amide nitrogen apparently is used in the synthesis of amino acids and proteins in the maturing plant. The young plants of Experiment I accumulated a rather high concentration of ammonia in their roots and in response to this abundant supply of ammonia synthesized amides in considerable amounts. The lower supply of ammonia to the tops was reflected in a much less vigorous amide synthesis there. The older plants of Experiment II accumulated less ammonia and synthesized a lower concentration of amides (analyzed in the protein fraction) than did the young plants, but during the 12 hour period of treatment there was a higher percentage increase of amides in their tops than in their roots from the ammonia supplied. The N^{15} concentration of the amide fraction in the roots of the young plants (Experiment I) is high relative to the other fractions, but this was not true for the amides elsewhere. Apparently their importance was largely confined to young root tissue high in ammonia nitrogen. The amide fractions are not as clear cut as might be desired, for the treatment of the tissue was such that partial hydrolysis may have occurred; the amide and ammonia fractions in Experiment II were not separated and were determined in the protein fractions only.

The dicarboxylic amino acids, glutamic and aspartic, contained high concentrations of N^{15}, indicating their rapid synthesis or turn over. In the leaf and stem tissue protein (Table II) glutamic acid was found to have an N^{15} concentration 7 times the average for the whole protein. A similar high order of reactivity of these two amino acids has been observed in the tissues of rats fed isotopically labeled ammonium citrate (8) and in tobacco plants furnished the ammonium ion (2). These data also show a lower order of reactivity for aspartic acid in tomato, of interest in view of the dominance of glutamine over asparagine in this species (9). The probable importance of oxalacetate and α-ketoglutarate in carbohydrate trans-

formations in the higher plants is now generally recognized. The rapid formation from ammonium nitrogen of the corresponding amino acids supports the suggestions of Chibnall (10) and Vickery and Pucher (11) concerning the importance of these α-keto acids in nitrogen metabolism.

Histidine was found to contain relatively less N^{15} (0.21 atom per cent N^{15} excess) than most of the other amino acids or fractions. Lysine showed a particularly low order of reactivity as indicated by an isotope content of only 0.07 atom per cent excess. An intermediate concentration of N^{15} was found in arginine, indicating that this amino acid is relatively more active metabolically than the other basic amino acids. The isotope concentration of the phosphotungstic acid filtrate, containing principally the mono-amino monocarboxylic amino acids, was also intermediate. Two copper salt fractions were isolated and similar isotope concentrations found in' each.

SUMMARY

Tomato plants were supplied for a short time with $(NH_4)_2SO_4$ labeled with N^{15}. Various nitrogenous fractions and amino acids were then isolated and analyzed for their concentration of N^{15}. Rapid absorption, translocation, and utilization of the ammonium ion were found to have occurred after 4 and 12 hours. All nitrogenous fractions or amino acids isolated contained significant excesses of N^{15}. Among the amino acids and amino acid fractions separated, glutamic acid had an outstandingly high concentration of N^{15}; aspartic acid also had a high level of N^{15}. In young plants the distribution of N^{15} indicated a considerable synthesis of amides, particularly in the roots where much ammonia was present. Of the compounds isolated the basic amino acids, histidine and lysine, had the lowest N^{15} concentration.

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ANTAGONISM OF AMINO ACIDS IN THE GROWTH OF LACTIC ACID BACTERIA*

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Inhibition of microbial growth by compounds closely related to essential metabolites has been demonstrated by many investigators (1–3). Early workers (4–7) showed that inhibitory effects of single amino acids could be reversed by altering the concentration of structurally related amino acids. Gladstone (8), using *Bacillus anthracis*, showed such a relationship among leucine, valine, and isoleucine and between threonine and serine. Threonine exerts an antagonistic action on the utilization of serine by *Lactobacillus arabinosus* (9). Using *Lactobacillus casei*, Feeney and Strong (10) demonstrated an inhibitory effect of aspartic acid, reversible by glutamine, glutamic acid, or asparagine.

While investigating a uniform medium for the microbiological determination of amino acids (11), certain difficulties were encountered with the glutamic acid and isoleucine assays with *Lactobacillus arabinosus*. A lag in the growth response curve was observed at the lower concentrations of these amino acids. The tubes containing samples, particularly in the case of isoleucine assays, did not show this lag to the same extent as the standard curves, resulting in marked downward drift of assay values. To alleviate the lag in the glutamic acid curve and to give a valid assay, a heavy inoculum and adjustment of the medium to pH 6 have been used (12). While this provided a satisfactory assay for glutamic acid, further work on the fundamental defect was indicated. As in previous studies (13, 12, 14, 15), when glutamic acid standard was replaced by glutamine (sterilized by filtration) the lag was absent. This indicated, as suggested previously (12, 14), that glutamic acid is utilized through glutamine and that the lag is the result of partial or complete failure of the small amounts of glutamic acid present to be amidated to glutamine.

In this study the mutual antagonism of the members of two groups of

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amino acids in the growth of *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* P-60 was investigated.

EXPERIMENTAL

The cultures and assay techniques used were the same as described previously (11), except that light inocula were used. To accentuate the lag in growth, the inocula were diluted until no turbidity was perceptible. In later experiments, the inocula were carefully standardized to a somewhat greater dilution than this by suspending the cells in sufficient sterile water to give a reading of 50 in an Evelyn colorimeter, with standard Evelyn tubes, and a 660 mμ filter against a water blank. 1 ml. of this suspension was then diluted to 200 ml. with sterile, distilled water and 1 drop was used to inoculate each 2 ml. of culture medium.

In most studies, 2 ml. volumes in 18 × 150 mm. culture tubes were used. In some of the glutamic acid experiments early growth in 10 ml. volumes, in Evelyn colorimeter tubes, was measured turbidimetrically against an uninoculated blank. In other work where a number of solutions had to be sterilized by filtration 0.2 ml. volumes were used. They were titrated electrometrically (16) after 60 to 72 hours of incubation.

Results

Effect of Other Amino Acids on Isoleucine Requirement—Inhibition indices (3) were determined for *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* with isoleucine as the metabolite and leucine and valine as the antagonists. Typical results are shown in Table I. The inhibition indices were not constant, but were of the same general magnitude over wide concentration ranges. In all cases increasing the concentration of the metabolite reversed the inhibition, indicating that it is competitive. DL-Valine and L-leucine were additive in antagonizing the growth of *Lactobacillus arabinosus* when isoleucine was the limiting amino acid. DL-Leucine was approximately half as effective as L-leucine as an antagonist of isoleucine, indicating that only the L isomer is involved; D-leucine was not tested. Decreasing the concentration of DL-valine and L-leucine in the basal medium from the normal level of 400 and 200 γ per 2 ml. tube to 100 and 50 γ, respectively, eliminated the lag.

Excessive amounts of methionine inhibited the growth of *Lactobacillus arabinosus* when isoleucine was the limiting amino acid. 10 mg. per tube of DL-methionine completely suppressed the growth of *Lactobacillus arabinosus* in the presence of 30 γ of DL-isoleucine. Alanine at 30 mg., or DL-serine or DL-threonine at 50 mg. per tube, caused no inhibition, indicating that the antagonism is not general for all amino acids (Table II). That these relationships are not peculiar to the medium being used in these studies was indicated by parallel experiments in which a medium typical of those currently employed by many other workers (17) was used.

TABLE I

Results of Typical Experiment Showing Effect of Valine and Leucine As Antagonists of Isoleucine for Lactobacillus arabinosus

DL-Isoleucine	DL-Valine	L-Leucine	Titer	Molar inhibition index*
γ per tube	γ per tube	γ per tube		
30	500	50	82	
30	1,000	50	72	
30	1,500	50	54	
30	2,000	50	6	45–60
200	15,000	50	192	
200	20,000	50	181	
200	25,000	50	102	
200	30,000	50	67	
200	45,000	50	13	
2000	15,000	50	195	
2000	75,000	50	179	135–200
6	100	100	9	
6	100	500	5	
30	100	200	105	
30	100	400	87	
30	100	800	53	
30	100	1000	14	27–33

* Based on concentrations of L forms of amino acids.

TABLE II

*Summary of Results of Studies of Antagonism of Amino Acids**

Limiting amino acid	Inhibiting amino acid	Molar inhibition index	
		L. arabinosus	*L. mesenteroides*
Isoleucine	DL-Valine	45–60	500–1000
	L-Leucine	30–40	75–250
	DL-Leucine	40–50	Not determined
	DL-Methionine	100–200	" "
	DL-Serine	None at 4500	" "
	DL-Threonine	" " 3500	" "
	DL-Alanine	" " 1400	
Leucine	DL-Isoleucine	450	None at 3000
	DL-Valine	2000	
	DL-Methionine	None at 1500	Not determined
	DL-Threonine	" " 5500	" "
	DL-Serine	" " 6200	" "
Valine	L-Leucine	360	None at 1800
	DL-Isoleucine	20–40	700
	DL-Methionine	<300	Not determined

* All based on concentrations of the L isomers.

When five organisms were used to determine isoleucine in acid-hydrolyzed casein, all except *Lactobacillus arabinosus* gave nearly identical values, with no drift. With *L. arabinosus* high values at the lower levels and low values in the upper part of the standard curve, *i.e.* a drift downward, were encountered. Thus, for example, values of 6.1 per cent with *Streptococcus faecalis* R, 6.0 per cent with *Leuconostoc mesenteroides*, 6.0 per cent with *L. delbrueckii*-3, and 6.1 per cent with *L. casei* were obtained. Values with *L. arabinosus* drifted from 10 to 5.6 per cent, indicating that the standard curve was below the "sample curve" at low levels and above it at the higher levels.

TABLE III

*Effect of Regular and Low Leucine and Valine Concentrations in Medium on Iso-leucine Assay of Casein Hydrolysate**

Sample weight	L. mesenteroides		L. arabinosus	
	Low	Regular	Low	Regular
mg.				
0.1	6.4	9.5	6.5	15.7
0.2	6.8	7.3	4.6	9.3
0.3	6.0	6.4	3.8	7.4
0.4	6.0	6.2	3.7	6.2
0.5	6.3	6.0	3.3	5.7
Average...... ...	6.3	7.1	4.4	8.9

* Regular levels were DL-valine 0.4 mg. per 2 ml. tube and L-leucine 0.2 mg. per 2 ml. tube. Low levels were one-fourth of the regular concentrations.

The effect of lowering the level of leucine and valine in the medium for the determination of isoleucine in another protein hydrolysate is shown in Table III. With regular concentrations of leucine and valine in the medium, there was drift in the values, particularly with *Lactobacillus arabinosus*. Decreasing the level of these amino acids eliminated the slight drift for *Leuconostoc mesenteroides*, giving assay values which compare well with those obtained with *L. delbrueckii*-3 (6.6 per cent). When *L. arabinosus* was the test organism, the drift was not eliminated by lowering the concentrations of leucine and valine in the medium, and much lower values were obtained. With *Leuconostoc mesenteroides*, inhibition of growth occurred in the lower portion of the curve when regular levels of leucine and valine were present in the medium, but the upper portion of the curve coincided with that obtained with low levels of these two amino acids (Curves 2 and 3, Fig. 1). With *L. arabinosus*, low levels of leucine and valine gave a standard curve elevated above that obtained with a medium containing

regular concentrations of leucine and valine, at all levels of isoleucine (Curves 1 and 4, Fig. 1). This indicated utilization of the D isomer of isoleucine by *L. arabinosus* at low levels of leucine and valine. To verify the variable utilization of D-isoleucine by this organism, when regular and low levels of leucine and valine were used, standard curves with L- and DL-isoleucine were prepared. With regular levels of leucine and valine the L and DL standard curves (Curves 1 and 2, Fig. 2) nearly coincided, except
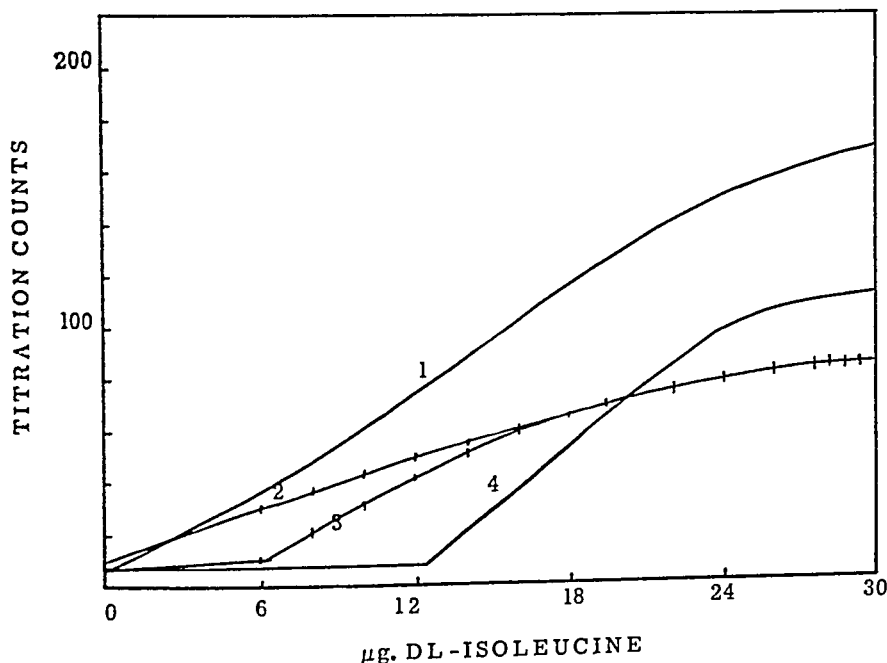
FIG. 1. Effect of leucine and valine concentrations on isoleucine standard curves. Curves 1 and 2, L-leucine 0.05 mg. and DL-valine 0.1 mg. per 2 ml. tube; Curves 3 and 4, regular levels, L-leucine 0.2 mg. and DL-valine 0.4 mg. per 2 ml. tube. Curves 1 and 4, *Lactobacillus arabinosus*; Curves 2 and 3, *Leuconostoc mesenteroides*.

in the upper portion. In this portion, the ratio of the concentrations of leucine and valine to isoleucine was less than in the lower portion of the curve, resulting in greater utilization of the D isomer. With low levels of leucine and valine present in the medium, the L and DL curves were not superimposable. The DL-isoleucine standard curve (Curve 4, Fig. 2) diverged upward from the L-isoleucine standard curve (Curve 3, Fig. 2), indicating that the D isomer was being utilized at nearly all concentrations of isoleucine. When D-isoleucine was added in increasing concentrations to a medium containing the L isomer at a level sufficient for half maximum growth (7.5 γ per tube), the titration values increased, indicating 10 per

cent activity at 10 γ per tube, and 19 per cent activity at 30 γ per tube. When D-isoleucine was used alone for preparing a standard curve, it showed no growth-promoting activity.
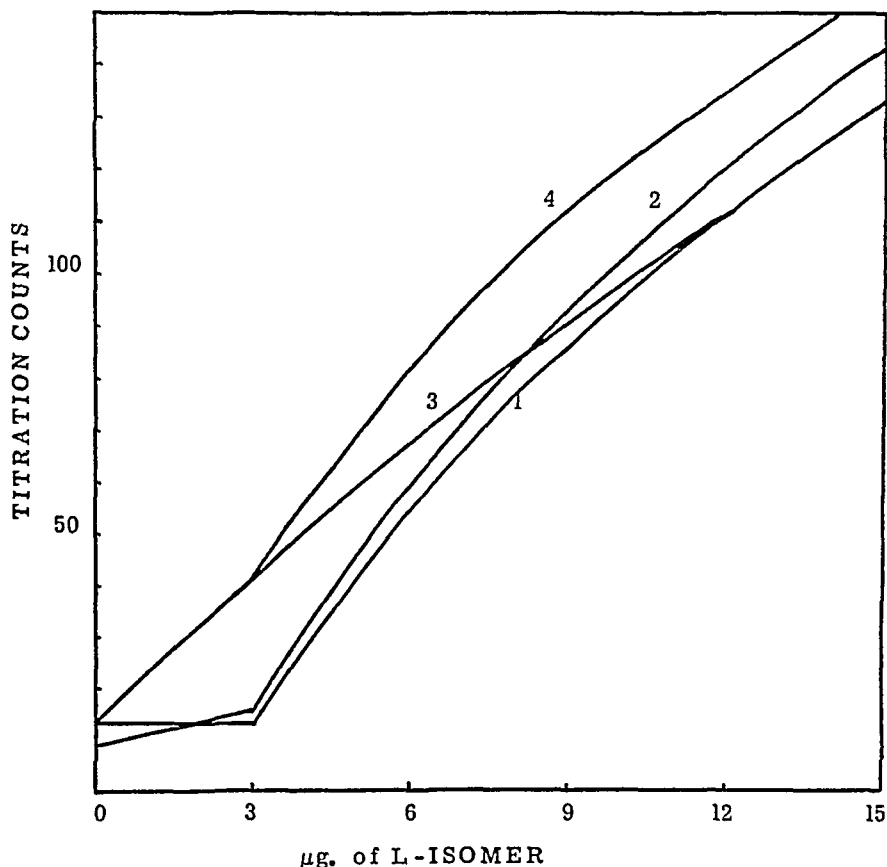
FIG. 2. Effect of regular and low leucine and valine on response of *Lactobacillus arabinosus* to L- and DL-isoleucine. Curve 1, L-isoleucine, regular leucine and valine; Curve 2, DL-isoleucine, regular leucine and valine; Curve 3, L-isoleucine, low leucine and valine; and Curve 4, DL-isoleucine, low leucine and valine.

During these studies the contamination of DL-isoleucine (Merck), presumably with alloisoleucine, was noted. The activity for *Leuconostoc mesenteroides* and *L. delbrueckii*-3 of this DL-isoleucine, as compared to a pure sample of L-isoleucine,[1] was only 39.5 per cent, instead of the 50 per cent expected for pure DL-isoleucine. This was also observed by Mr. F. A. Wacher of Merck and Company, Inc., and was reported by Smith and Greene (18).

[1] Kindly supplied by Dr. D. G. Doherty of this laboratory.

Effect Of Other Amino Acids on Leucine and Valine Requirements—With leucine limiting, the concentrations of either DL-valine or DL-isoleucine required to inhibit growth of *Lactobacillus arabinosus* were nearly the same. The inhibition indices (Table II) indicate that this organism is less sensitive to these antagonisms when leucine is limiting than when isoleucine is the limiting nutrient. Methionine, threonine, and serine did not show the antagonism at the high concentrations used.

When valine was the limiting amino acid for the growth of this organism, DL-isoleucine caused inhibition at lower concentrations than did L-leucine. From Table II it is evident that leucine is antagonized less by isoleucine and valine than is valine by isoleucine and leucine. These differences account for the occasional slight lag in the valine standard curves with this medium and this organism and the absence of such lags in the leucine curves with similar assay conditions. The presence or absence of a lag from one valine assay to another is probably a result of slight variations in the weight and age of the inoculum. Methionine antagonized the utilization of limiting quantities of valine.

An antagonizing action by isoleucine for *Leuconostoc mesenteroides* with low concentrations of leucine could not be shown. Growth could be inhibited, however, by large amounts of isoleucine when the valine level was limiting. The relatively lower susceptibility of *Leuconostoc mesenteroides* to these imbalances is probably one reason why this organism is now so widely used for amino acid assays.

Glutamic Acid-Aspartic Acid Relationship—The general lag in the growth of *Lactobacillus arabinosus* when glutamic acid was limiting was evidenced in the lower portion of the standard curve, but occasionally extended over one-half of the range. Adjustment of the medium to pH 6 together with the use of a heavy inoculum (12) was a practical solution. Lyman *et al.* (14) added small amounts of glutamine to prevent this lag. Replacement of aspartic acid by asparagine has been reported by Baumgarten *et al.* (15) to relieve the lag, but when asparagine was added to our medium already containing aspartic acid, the lag was accentuated. This suggested that aspartic acid or asparagine might be the active substance causing the lag in the growth curve. When the concentration of aspartic acid in the medium was progressively lowered, the lag was diminished until it was completely eliminated at 40 γ per tube (Fig. 3). A 10- to 20-fold greater concentration of asparagine than aspartic acid was necessary to elicit an equivalent lag in growth. Table IV shows the effect of asparagine and aspartic acid on the metabolism of glutamic acid by *L. arabinosus*. These data show an inhibition index of 400 for asparagine and approximately 20 for L-aspartic acid. Asparagine exerts a stimulatory effect on the growth of this organism (Table IV) as shown by slightly higher titrations

TABLE IV

Effect of Asparagine and Aspartic Acid on Metabolism of Glutamic Acid for Lacto-bacillus arabinosus*

Concentration of glutamic acid	No aspartic acid	Concentration			
		L-Asparagine, 2 mg.	DL-Asparagine, 2 mg.	L-Aspartic acid, 2 mg.	DL-Aspartic acid, 2 mg.
γ					
0	3	3	4	5	5
5	20	7	4	5	4
10	30	25	20	5	5
25	43	56	56	5	5
80	86	102	98	5	5
100	96	108	101	11	72

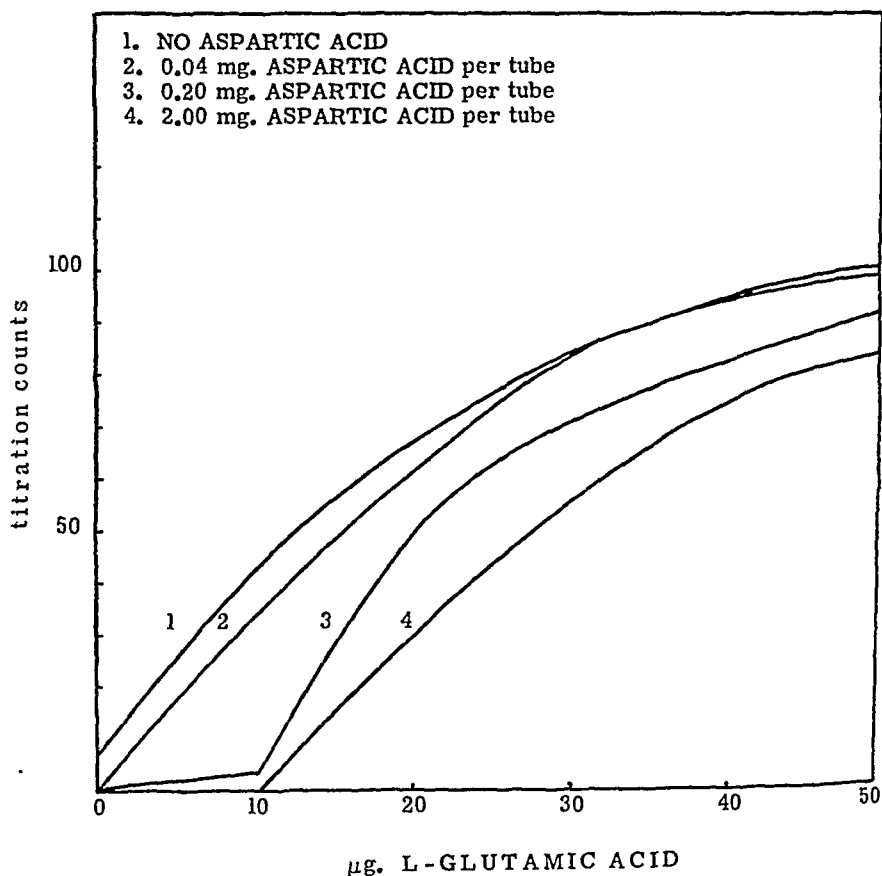* Final pH of media after autoclaving 7.1. Each count represents 0.05 ml. of 0.04 N NaOH.

FIG. 3. The effect of L-aspartic acid and L-asparagine concentration on the response of *Lactobacillus arabinosus* to glutamic acid.

for the medium containing added asparagine.   Three different samples of asparagine (one of racemate and two of the L form) were used, at the same concentration, to test the possibility that the lag was due to aspartic acid as a contaminant of the asparagine.   All samples gave the same degree of lag in growth.   It is unlikely that three different samples would contain equal amounts of aspartic acid.   From these data, it appears that the antagonistic action is due to the asparagine *per se*.

With aspartic acid limiting, high glutamic acid concentrations had no effect on the growth of *Leuconostoc mesenteroides*.

TABLE V

Inhibition of Growth of Lactobacillus arabinosus by Bicarbonate and L-Aspartic Acid in Presence of 0.425 × 10⁻³ M Glutamic Acid or Glutamine

NaHCO₃	L-Aspartic acid	Glutamic acid*	Molar inhibition index	Glutamine*	Molar inhibition index
0	0	137		120	
10.9×10^{-3}	0	133		114	
23.8×10^{-3}	0	22	56	35	56
35.6×10^{-3}	0	2	83	1	83
47.6×10^{-3}	0	0		0	
59.5×10^{-3}	0	0		0	
0	0	147		130	
0	7.5×10^{-3}	21	19	135	
0	15.0×10^{-3}	8	35	135	
0	22.5×10^{-3}	8		136	
0	30.0×10^{-3}	7		136	
0	37.7×10^{-3}	7		137	

* The values represent titration counts, each count equivalent to 0.05 ml. of 0.04 N NaOH.

During these studies, Waelsch *et al.* (13) reported inhibition of the growth of *L. arabinosus* by oxalacetate and sodium bicarbonate.   To determine whether these antagonisms had a common basis, inhibition indices were determined for L-aspartic acid and sodium bicarbonate.   Table V shows the results of one such experiment.   Although inhibition by aspartic acid was not observed when glutamine was present, glutamine had no effect on the bicarbonate inhibition.   The molar inhibition index for aspartic acid was considerably lower than that for sodium bicarbonate.

DISCUSSION

The amino acid requirements of many lactic acid organisms have been studied extensively in the search for more specific and reliable assays, but little has been reported on the effect of high concentrations of amino acids.

The policy has been to add an excess of all required nutrients, with the exception of the one being assayed. The results reported here and those of Meinke and Holland (9) indicate that the amino acids should also be present in correct proportions to avoid imbalances. Addition of an inhibiting amino acid with the sample might result in growth suppression in the sample tubes not encountered in the standard tubes, with resulting drift and invalidation of the assay. This might dictate a medium containing little more of such amino acids than the bacteria need for maximum growth. When such a medium is used, however, the percentage difference in concentration in sample tubes and standard tubes is very much greater and might prove quite significant when proteins of other than average composition are assayed. A safer procedure appears to be that of maintaining moderately high concentrations of all constituents, so that the percentage change in composition of the fermentation liquid is affected only slightly by addition of the sample. In cases in which difficulties arise, as evidenced by lag, drift in assay values, poor recovery of added amino acid, or inconsistent values, other organisms less sensitive to such imbalances should be employed. The use of L standards is advisable to eliminate possible activity of the D isomer.

With an isoleucineless strain of *Neurospora crassa*, it has been demonstrated that β-methyl-α-ketovaleric acid, the keto derivative of isoleucine, inhibits the conversion of the keto acid analogue of valine to valine (19). This may be due to saturation of the surface of the enzyme which reductively aminates this keto acid to valine by the structurally related isoleucine derivative. A similar mechanism may account for the relationships described here. Isoleucine may be utilized as a peptide or a similar derivative and its incorporation into such an active intermediate may be mediated by an enzyme which is effectively blocked by the homologous or isomeric amino acids.

Lyman *et al.* (20) have recently reported that the D isomer of isoleucine is utilized by *Lactobacillus arabinosus* when vitamin B$_6$ is present in the form of pyridoxamine; utilization was greater when leucine was present at 0.4 mg. than at 2.0 mg. per 10 ml. tube. The concentration of leucine used in our studies (11) is one-half the level reported to inhibit utilization of D-isoleucine. The low levels used in these studies are comparable to those found by Lyman *et al.* (20) to enhance utilization of the D form. The anomalous results obtained in assaying samples, with a medium containing high concentrations of valine and leucine and in the presence of pyridoxal, when DL-isoleucine was used for the standard curve, can largely be explained by a combination of the antagonism described here and the variable availability of D-isoleucine for *Lactobacillus arabinosus* shown by Lyman *et al.* (20).

When aspartic acid-glutamic acid ratios were calculated for the data of Hac *et al.* (12), inhibition indices ranged from 30 to 200, depending on pH, weight of inoculum, length of incubation, and whether ammonium salts were present. The inhibition indices found here essentially confirm their work.

Waelsch *et al.* (13), using a 20 hour turbidimetric assay, found that at a concentration of 0.82×10^{-3} M glutamic acid, 24×10^{-3} M oxalacetate at pH 5.7 or 9.5×10^{-3} M $NaHCO_3$ at pH 7.4 would inhibit completely the growth of *Lactobacillus arabinosus*. The inhibition due to oxalacetate could be reversed by a 4-fold increase in concentration of glutamic acid or by 7.0×10^{-5} M glutamine while $NaHCO_3$ inhibition was reversed by a 3-fold increase of glutamic acid or by 2.7×10^{-6} M glutamine. They attributed the inhibition to carbon dioxide, which appeared to prevent the amidation of glutamic acid to glutamine. The competitive aspartic acid-glutamic acid growth inhibition obtained with *L. arabinosus* appears to be a function of the enzyme system which converts glutamic acid to glutamine. Aspartic acid or asparagine may inhibit this reaction by competing with glutamic acid for the enzyme catalyzing this conversion. In our studies, glutamine reversed aspartic acid inhibition, but failed to overcome bicarbonate inhibition. From these results, it appears that these inhibitors do not act by a common mechanism. Aspartic acid is a more effective inhibitor than sodium bicarbonate. Interpretation of the data of Waelsch *et al.* (13) is complicated by the presence of aspartic acid in the medium. Calculation of the inhibition indices showed that asparagine, methionine sulfoxide (21), $NaHCO_3$ (13), oxalacetate (13), and aspartic acid were 200 to 400, 75, 30, 30, and 20, respectively. In these studies, the index for sodium bicarbonate was approximately 50.

By replacing the aspartic acid in the uniform medium (11) for amino acid assays by one-half as much L- or DL-asparagine, a dose-response curve to glutamic acid is obtained which is much more nearly linear and whose slope is largely independent of the size of the inoculum. Such a modified medium has proved highly satisfactory for the determination of glutamic acid.

SUMMARY

1. The metabolism of *Lactobacillus arabinosus* is affected by the balance of concentrations of leucine, isoleucine, valine, and methionine present in the medium.

2. When isoleucine was the limiting amino acid, high concentrations of leucine, valine, and methionine in decreasing order of effectiveness caused inhibition of growth. Alanine, threonine, and serine did not cause inhibition when added at high levels, indicating reaction specificity.

3. When leucine was limiting, isoleucine inhibited growth more than valine and when valine was limiting, isoleucine inhibited growth more than leucine.

4. The growth of *Leuconostoc mesenteroides* P-60 was also affected by imbalances of these amino acids. However, the concentrations necessary to inhibit this organism were approximately 5 times greater than those required to inhibit *Lactobacillus arabinosus*.

5. The growth of *Lactobacillus arabinosus*, with glutamic acid limiting, was inhibited by aspartic acid or asparagine; the former was the more effective antagonist. It appears that these amino acids inhibit by preventing the small amounts of glutamic acid present from being amidated to glutamine. This organism is much less sensitive to this inhibition below pH 7.

6. The uniform medium of Henderson and Snell (11) should be modified for the glutamic acid assay when *Lactobacillus arabinosus* is used by replacing the aspartic acid with asparagine at a lower concentration.

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REACTION OF *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE WITH *l*-ASCORBIC ACID AND OTHER ENEDIOL COMPOUNDS, WITH A NOTE ON THE ISOLATION OF DEHYDRO-*l*-ASCORBIC ACID

By RADWAN MOUBASHER

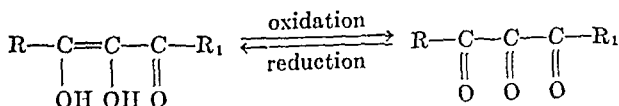(*From the Faculty of Science, Fouad 1st University, Cairo, Egypt*)

When solutions of *peri*-naphthindan-2,3,4-trione hydrate (1) (II) and ascorbic acid (I), reductone, or dihydroxymaleic acid are mixed at a constant temperature of 45° for about 6 hours, or heated on a boiling water bath for 3 minutes and then cooled, definite silky red crystalline precipitates are formed. These precipitates, which proved to be dihydroxy-*peri*-naphthindenone (III), are similar to the compound prepared by Errera (2), using the action of hydrogen sulfide on *peri*-naphthindan-2,3,4-trione hydrate, and have the same melting point; the mixed melting point was not depressed on addition of an authentic specimen. The same characteristic deep blue coloration is also obtained when the substance is treated with sodium hydroxide solution.

l-Ascorbic acid, reductone, and dihydroxymaleic acid belong, according to their structures and properties, to the class of "reductones," which are characterized by the following constitution:

$$R-\underset{\underset{OH}{|}}{C}=\underset{\underset{OH}{|}}{C}-\underset{\overset{\|}{O}}{C}-R_1$$
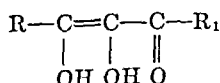
The formulae of these compounds contain a common grouping with that of reductone, which explains their similar behavior, and all compounds of this class form reversible oxidation-reduction systems.

$$R-\underset{\underset{OH}{|}}{C}=\underset{\underset{OH}{|}}{C}-\underset{\overset{\|}{O}}{C}-R_1 \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} R-\underset{\overset{\|}{O}}{C}-\underset{\overset{\|}{O}}{C}-\underset{\overset{\|}{O}}{C}-R_1$$

It follows from these facts that the oxidation of *l*-ascorbic acid by *peri*-naphthindan-2,3,4-trione hydrate stops at the stage of dehydro-*l*-ascorbic acid (IV), since a mixture of dehydro-*l*-ascorbic acid and *peri*-naphthindan-2,3,4-trione hydrate gives no precipitate of dihydroxy-*peri*-naphthindenone. During this reaction and the period of heating at about 45°, there is evolution of carbon dioxide. This is due to decarboxylation of the dehydro-*l*-ascorbic acid after hydrolysis of the lactone bridge, forming *l*-

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xylo-2,3-diketohexonic acid (V) as an intermediate product and finally *l*-xylosone (VI) (*cf.* West and Rinehart (3), Koppanyi, Vivino, and Veitch (4)).

In the case of reductone, the stage of oxidation stops at the formation of mesoxalaldehyde, which is identified in the form of its diphenylhydrazone. Dihydroxymaleic acid is oxidized to diketotartaric acid, which is identified in the form of its osazone.

$$
\begin{array}{l}
O{=}C{-} \\
HO{-}C \\
HO{-}C \quad O \\
HC{-} \\
CHOH \\
CH_2OH
\end{array}
\quad (I)
\;+\;
(II)
\;\longrightarrow
$$

(I)   (II)

$$
(III) \;+\; (IV) \;\xrightarrow{\;HOH\;}
$$

(III)   (IV)

$$
\begin{array}{l}
COOH \\
C{=}O \\
C{=}O \\
CHOH \\
CHOH \\
CH_2OH
\end{array}
\quad\xrightarrow{\;-CO_2\;}\quad
\begin{array}{l}
CHO \\
C{=}O \\
CHOH \\
CHOH \\
CH_2OH
\end{array}
$$

(V)   (VI)
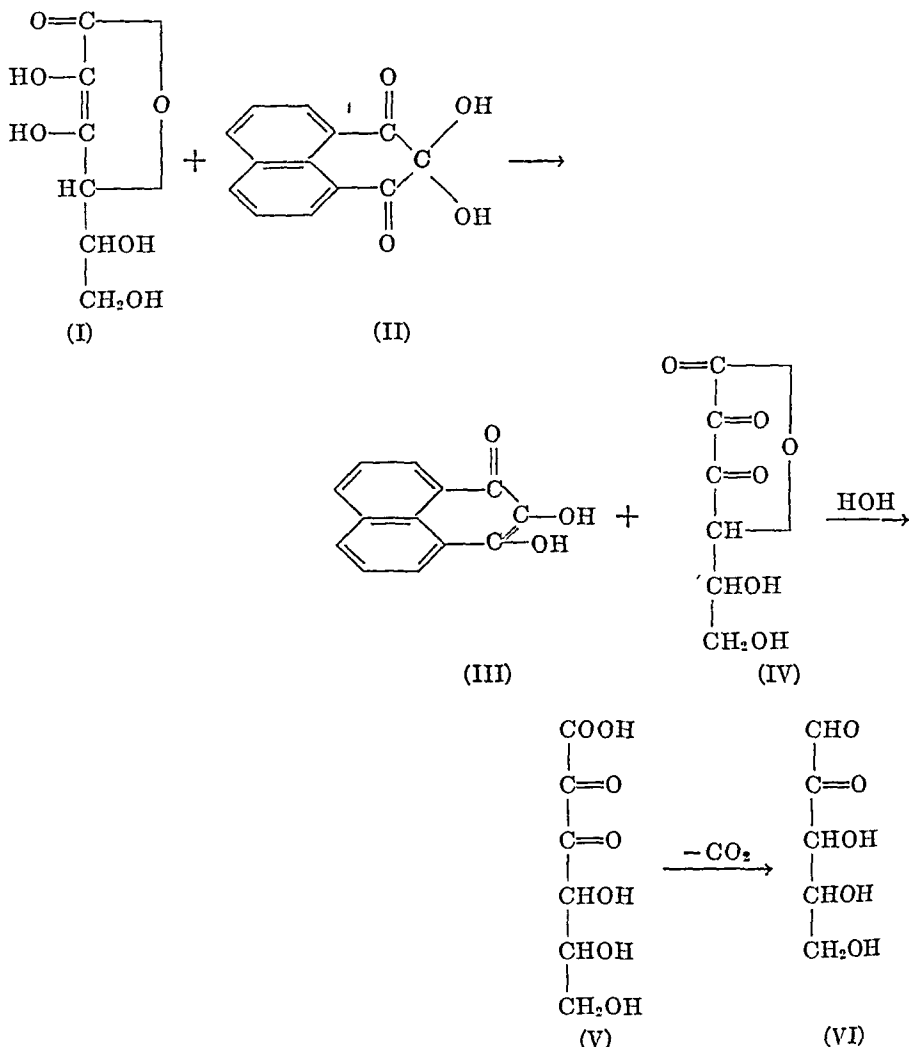
The chemistry of dehydro-*l*-ascorbic acid, the primary reversible oxidation product of vitamin C, has hitherto been mainly investigated by

utilizing solutions of the freshly oxidized vitamin. The labile nature of this substance has, however, prevented its isolation hitherto in a pure state, although Karrer, Salomon, and Schöpp (5) and Crook and Morgan (6) have prepared it in a pure but amorphous form. Kenyon and Munro (7) found recently that when the solid product obtained by the method of Hirst and Woodward is intensively dried and dissolved in ethanol at room temperature the solution on standing deposits a crystalline colorless compound which has the characteristics of anhydrous dehydro-*l*-ascorbic acid.

During the action of *peri*-naphthindan-2,3,4-trione hydrate (II) on freshly crystallized *l*-ascorbic acid (I) in aqueous solutions at 20° for 1 hour, it has been found that after separation of dihydroxy-*peri*-naphthindenone (III) and concentration of the filtrate to dryness, followed by treatment with ethanol at 0°, anhydrous dehydro-*l*-ascorbic acid (IV) is isolated in a crystalline state.

It has also been found *in vitro* that dehydro-*l*-ascorbic acid reacts on α-amino acids at the boiling point of water with the formation of ammonia, carbon dioxide, and the corresponding aldehydes with 1 carbon atom less (*cf.* von Euler, Karrer, and Zehender (8), Abderhalden (9)). This degradation reaction has been carried out under physiological conditions of temperature and pH. According to this evidence, it appears reasonable to believe that vitamin C deficiencies which occur quite often may be due to intestinal destruction of the vitamin, partially by hydrolysis followed by decarboxylation of the dehydro-*l*-ascorbic acid and partially to its action on the α-amino acids. This will explain among other reasons the relatively high requirements of this vitamin on the weight basis compared to the daily needs of man and animals for the other vitamins.

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with l-Ascorbic Acid— 0.5 gm. of ascorbic acid mixed with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of water is heated in a boiling water bath for 3 minutes and quickly cooled; a red silky crystalline precipitate is obtained, which is filtered off, washed several times with cold water, and then dried; m.p. 258°; yield 0.42 gm. This substance proved to be dihydroxy-*peri*-naphthindenone by mixed melting point with an authentic specimen prepared according to Errera (1, 2) and also by the characteristic intense blue color which is obtained on treatment with sodium hydroxide solution.

$C_{13}H_8O_3$.   Calculated, C 73.5, H 3.7; found, C 73.6, H 3.8

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with l-Ascorbic Acid at 45°—0.5 gm. of *l*-ascorbic acid is dissolved in 10 cc. of water and mixed with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate dissolved in 15 cc. of water. The vessel containing the mixture is arranged so that a continual current of pure hydrogen is passed through it, and it is

placed on a constant bath of 40° for 10 hours.   It is connected to a bubbler containing barium hydroxide to provide for titration of the amount of carbon dioxide.   From 0.5 gm. of ascorbic acid treated with 0.6 gm. of *peri*-naphthindan-2,3,4-trione hydrate at 45°, 0.5 gm. of carbon dioxide was evolved in 6 hours.   The solid dihydroxy-*peri*-naphthindenone is separated by filtration and the clear filtrate is concentrated *in vacuo* to a syrupy liquid.   This is treated with a phenylhydrazine mixture (0.4 gm. of phenylhydrazine hydrochloride mixed with 0.6 gm. of sodium acetate and 4 cc. of distilled water).   *l*-Xylosazone has been obtained in delicate yellow needles and melts at 164°, with decomposition.   This is proved by the melting point and mixed melting point of the osazone.

Reaction of peri-Naphthindan-2,3,4-trione Hydrate with Dehydro-l-ascorbic Acid—0.5 gm. of ascorbic acid dissolved in 10 cc. of distilled water is treated with iodine-potassium iodide solution (1.4 gm. of iodine in 2.5 gm. of potassium iodide dissolved in 10 cc. of water) until it is transformed into dehydro-*l*-ascorbic acid.   The hydrogen iodide is removed by the addition of silver carbonate and filtration.   0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate is added with 25 cc. of water and heated in a boiling water bath for 3 minutes and then cooled; no reaction takes place. The whole solution is concentrated *in vacuo* and then allowed to cool, when a yellowish crystalline precipitate is obtained, which proved to be *peri*-naphthindan-2,3,4-trione hydrate by melting point, 273° with decomposition, and mixed melting point determinations and by color tests with sodium hydroxide solution.   The same experiment has been repeated with the same amounts in the presence of a current of pure hydrogen.   The reaction mass was placed at 45° for about 6 hours and the amount of CO_2 has been calculated as above and found to correspond to 0.4 gm.

Action of peri-Naphthindan-2,3,4-trione Hydrate with Reductone—0.3 gm. of reductone (*cf.* von Euler and Martius) (10)) with 0.6 gm. of pulverized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of distilled water is heated over a flame for 3 minutes and cooled; a red silky crystalline precipitate is obtained which is filtered off and recrystallized from ethyl alcohol; the melting point, 258°, and mixed melting point determinations with an authentic specimen proved the compound to be dihydroxy-*peri*-naphthindenone.   Yield, 0.35 gm.   The filtrate is concentrated· *in vacuo* and treated with the phenylhydrazine mixture.   The red substance which separates is recrystallized from methyl alcohol to give a red crystalline substance melting at 176°; the substance proved to be the diphenylhydrazone of mesoxalaldehyde by melting point and mixed melting point determinations with material prepared according to Pechmann (11).

Action of peri-Naphthindan-2,3,4-trione Hydrate with Dihydroxymaleic Acid—0.4 gm. of dihydroxymaleic acid (12) is mixed with 0.6 gm. of pul-

verized *peri*-naphthindan-2,3,4-trione hydrate in 25 cc. of distilled water and heated over a water bath at 45° for some hours and then cooled. A red, silky, crystalline precipitate obtained was filtered off and recrystallized from ethyl alcohol, forming red needles of melting point 258° which was not depressed when the substance was mixed with dihydroxy-*peri*-naphthindenone. Yield, 0.2 gm. The mother liquor of the reaction mass after separation of the red substance was concentrated *in vacuo* to a syrupy liquid which was chilled in ice for 24 hours. A colorless crystalline substance was obtained, melting at 114°; the mixed melting point was not depressed on admixture with diketotartaric acid. When this was treated with the phenylhydrazine mixture, orange crystals contaminated with impurities were obtained. By fractional crystallization uniform orange needles were separated, m.p. 200°. This osazone has been identified as the phenylhydrazone of diketotartaric acid by melting point and mixed melting point determinations (*cf.* Ziegler and Lacher (13)). The yield was very poor. This is due to the partial decomposition of dihydroxymaleic acid at 50–60°, with liberation of carbon dioxide and the formation of glycolaldehyde (*cf.* Fenton (14)).

Isolation of Dehydro-l-ascorbic Acid (IV)—3 gm. of pure *l*-ascorbic acid (I) dissolved in 25 cc. of distilled water were shaken with 3.5 gm. of *peri*-naphthindan-2,3,4-trione hydrate (II) in 150 cc. of distilled water at 20° for 1 hour. A reddish color first formed, followed by precipitation of red, silky, crystalline needles of dihydroxy-*peri*-naphthindenone (III). The whole mass was cooled at 0° and filtered off. When the filtrate was concentrated at 30° and 4 mm., a viscous yellowish syrup was obtained; this was dried *in vacuo* at 50° for 20 minutes. The resultant glassy substance was then left under a vacuum for some hours until of constant weight. This solid mass was powdered and shaken with 10 cc. of absolute alcohol and kept at 0° for 24 hours; anhydrous dehydro-*l*-ascorbic acid (IV) was obtained in fine colorless crystalline needles, m.p. 220° with decomposition, which were identified by mixed melting point determinations with an authentic sample prepared according to Kenyon and Munro (7). Yield, 0.8 gm.

Action of Dehydro-l-ascorbic Acid on Phenylaminoacetic Acid under Physiological Conditions of Temperature and pH—Dehydroascorbic acid (0.5 gm.), prepared by the method mentioned above, and phenylaminoacetic acid (0.3 gm.) are placed with water (75 cc.) in a flask fixed in a thermostat adjusted at 37°. The flask is provided with a delivery tube dipping into a solution of phenylhydrazine hydrochloride (0.4 gm. in 30 cc. of alcohol). After 72 hours, the contents of the reaction flask which had a strong odor of benzaldehyde are subjected to distillation under reduced pressure, carbon dioxide being bubbled through. The distillation is

controlled in such a way that a thermometer dipping into the reaction mixture does not rise over 37°; the receiver is cooled in an ice-salt mixture and connected to a trap containing the phenylhydrazine mixture mentioned above to retain any benzaldehyde which might have escaped. The distillate (about 50 cc.) is treated with the same phenylhydrazine mixture. Benzaldehyde phenylhydrazone was obtained which melted at 158° and showed no depression in the mixed melting point when mixed with an authentic specimen. Yield, 0.1 gm.

SUMMARY

1. Ascorbic acid, reductone, and dihydroxymaleic acid react with *peri*-naphthindan-2,3,4-trione hydrate at 45° for a long time, giving dihydroxy-*peri*-naphthindenone.

2. Ascorbic acid is oxidized to dehydroascorbic acid, followed by hydrolysis and decarboxylation with the formation of *l*-xylosone.

3. Reductone and dihydroxymaleic acid give respectively mesoxalaldehyde and diketotartaric acid, identified by their phenylhydrazone derivatives.

4. Dehydroascorbic acid has been isolated in a pure crystalline state.

5. Dehydroascorbic acid has been found to undergo Strecker degradation under physiological conditions of temperature and pH.

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THE METABOLISM OF 4-DIMETHYLAMINOAZOBENZENE BY RAT LIVER HOMOGENATES*

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The intact rat metabolizes the hepatic carcinogen 4-dimethylaminoazobenzene in several ways. Small amounts of the ingested dye and two demethylated derivatives, 4-monomethylaminoazobenzene and 4-aminoazobenzene, have been found in the liver and excreta (1). The latter two dyes and an unidentified aminoazo dye also occur in the liver firmly combined with protein; these bound dyes appear to be intimately associated with the carcinogenic process induced by the parent dye (2). Other tissues contain only 4-aminoazobenzene; this is also the only dye detectable in the blood and it is found entirely in the sedimented blood cells (1). In the urine approximately 50 per cent of the ingested dye can be accounted for in the form of two conjugated amines, p-phenylenediamine and p-aminophenol; small amounts of several other monophenylamines and demethylated hydroxyazo dyes are also present (3, 4). Thus 4-dimethylaminoazobenzene is subject to at least three metabolic reactions in the rat: demethylation of the dimethylamino group, hydroxylation, principally at the 4′ position, and reductive cleavage of the azo linkage. The exact sequence of these reactions and the extent to which each occurs are unknown.

The only previously published work on the metabolism of 4-dimethylaminoazobenzene by tissue *in vitro* demonstrated that this dye is destroyed by surviving rat liver slices (5); no metabolites of the dye were found in these reactions. The present communication deals with the metabolism of this carcinogen in fortified rat liver homogenates. In these reaction mixtures it has been possible to demonstrate that stepwise demethylation of the dye also occurs *in vitro* and that a new metabolite, 4′-hydroxy-4-dimethylaminoazobenzene, is formed by hydroxylation of the parent dye. The participation of several cell constituents, particularly diphosphopyridine nucleotide (DPN), in these reactions has also been studied.

Methods

Young adult Sprague-Dawley rats maintained on a grain diet were killed by decapitation and liver samples transferred immediately to ice-

cold isotonic KCl. In later experiments it was found that higher activity and substrate response were obtained with a preliminary fasting period of 18 to 24 hours. 10 per cent homogenates were prepared in isotonic KCl containing 8.0 ml. of 0.02 M K_2CO_3 per liter (6). All reactants were maintained at 0° prior to incubation.

A typical reaction mixture contained the following ingredients, each adjusted to pH 7.4 in a final volume of 3 ml.: 0.2 to 0.4 ml. of 10 per cent homogenate, 0.4 ml. of 0.03 M hexose diphosphate,[1] 0.1 ml. of 1 per cent diphosphopyridine nucleotide[1] (65 per cent assay), 0.2 ml. of 0.6 M nicotinamide, 0.1 ml. of 0.1 M $MgCl_2$, 0.4 ml. of 0.5 M KCl, and 0.5 ml. of 0.1 M K_2HPO_4-KH_2PO_4 buffer at pH 7.4. The 4-dimethylaminoazobenzene (20 to 50 γ) was customarily added last in 0.1 ml. of aldehyde-free 95 per cent ethanol. The most accurate addition and best dispersion of the dye were obtained by discharging the dye solution from a micro blow pipette. The mixtures were incubated in open 25 ml. Erlenmeyer flasks with mechanical shaking in a water bath held at 37.5°.

Estimation of Over-All Destruction of Dye—A rapid estimation of the residual dyes in the reaction mixtures was made by adding 3 ml. of a 20 per cent solution of trichloroacetic acid in 1:1 acetone-ethanol. This reagent precipitated the protein, extracted the adsorbed dyes, and developed the characteristic acid color (7, 8) of the dyes. The dye solutions were diluted to the proper optical density with equal parts of the trichloroacetic acid reagent and water. After light centrifugation the optical density of the solutions was determined at 520 mμ in a Cenco-Sheard spectrophotelometer adapted to the use of matched 13 mm. $\times$ 100 mm. Pyrex culture tubes. The solutions obeyed Beer's law up to at least 2.9 γ per ml. The tissue blanks, which were of low optical density, were deducted and the residual dyes expressed as equivalents of 4-dimethylaminoazobenzene. This rapid method gave reproducible results at the low tissue concentrations used (20 to 50 mg. per 3.0 ml. of reaction mixture).

Fractionation and Estimation of Possible Azo Metabolites of 4-Dimethyl-aminoazobenzene—Methods were devised by which the basic dyes, 4-dimethylaminoazobenzene, 4-monomethylaminoazobenzene, 4-aminoazobenzene, and the 4'-hydroxy derivatives of these dyes could be separately determined in the reaction mixtures. Recrystallized and chromatographically pure samples of the dyes prepared in this laboratory (9) were used. For fractionation of these possible metabolites the volume of the reaction mixture was doubled and incubated in open 50 ml. Erlenmeyer flasks. The reactions were stopped by the addition of approximately 0.5 gm. of solid $BaCl_2$ and 6.0 ml. of acetone. After standing for 20 to 40 minutes to com-

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[1] The hexose diphosphate, DPN, and ATP were generously supplied by Dr. G. A. LePage.

plete the extraction of the dyes from the precipitate, 8.0 ml. of benzene were added and the mixture shaken vigorously for $\frac{1}{2}$ to 1 minute. The basic and hydroxy dyes were thus extracted quantitatively into the acetone-benzene phase. Any emulsions that formed were broken by centrifugation in stoppered tubes and a 5 ml. aliquot of the upper phase was taken for analysis. This aliquot represented 41 per cent of the dyes in the reaction mixture. For uniformity of expression, the results are reported as the dye present in 3.0 ml. of reaction media.

The basic dyes were measured in the extract by removing the solvent *in vacuo* and redissolving the residue in Skellysolve B.[2] The dyes were separated from one another by chromatographing this solution on a 4 mm. × 100 mm. column of activated alumina. Each dye was separately eluted and determined by its color in strong acid as previously described (7).

Since the hydroxy dyes were adsorbed on the top of the alumina column and could not be quantitatively eluted, a separate 5 ml. aliquot of the initial extract was used for the estimation of these dyes. The solvent was removed *in vacuo* and the residue taken up in 5 ml. of Skellysolve B; this addition was followed by 5 to 10 ml. of 7 N HCl. The mixture was stirred mechanically for 1 minute to transfer all of the azo dyes to the acidic phase. The supernatant layer of Skellysolve B containing interfering tissue material was then decanted and a second extraction with this solvent performed. The residual solvent was removed under reduced pressure and the contents neutralized to approximately pH 3.0 and buffered by the addition of 1 ml. of 3 M KH_2PO_4 at pH 3.0. Exactly 6.0 ml. of benzene were added and the contents shaken for $\frac{1}{2}$ to 1 minute or until no acid color was visible in the aqueous phase. A 5 ml. aliquot of the extract, representing 34 per cent of the hydroxy dyes, was taken to dryness, redissolved in 0.1 to 0.2 ml. of benzene, and diluted with 2 ml. of Skellysolve B; this solution was resolved by chromatographic adsorption.

Hyflo Super-Cel (Johns-Manville) which had been washed with methanol and dried at 37° for 12 to 15 hours proved to be an excellent adsorbent for the chromatographic separation of the hydroxylated dyes. The basic dyes present in the extracts were readily eluted with Skellysolve B, and the hydroxy dyes formed slowly moving zones which were eluted successively with Skellysolve B containing 0.3 per cent of isopropanol. The dyes elute in an order similar to that of the basic dyes from alumina: 4'-hydroxy-4-dimethylaminoazobenzene > 4'-hydroxy-4-monomethylaminoazobenzene > 4'-hydroxy-4-aminoazobenzene. The N-methyl hydroxy dyes were extracted into 2 N HCl and yielded purple solutions with absorption maxima at 545 mμ and 535 mμ respectively for the dimethyl and monomethyl compounds. The 4'-hydroxy-4-aminoazobenzene was extracted into 7 N HCl

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[2] A commercial petroleum ether, b.p. 66–68°.

and yielded a yellow solution with an absorption maximum at 465 mμ. Experiments with 1 γ of either methylated dye proved that recoveries of 90 to 93 per cent from tissue could be consistently achieved. Due to the higher water solubility of 4′-hydroxy-4-aminoazobenzene, the recoveries of 2 γ quantities of this dye averaged 70 per cent. All of these dyes obeyed Beer's law in acid solution.

Attempts to use adsorption on Super-Cel to effect an initial separation of the hydroxy dyes from the basic dyes failed, since poor recoveries of the two basic dyes, 4-monomethylaminoazobenzene and 4-aminoazobenzene, occurred during the extraction of the dyes from acid solution at pH 3 with benzene. These conditions were required for the efficient extraction of the amphoteric hydroxyaminoazo dyes.

TABLE I

Effect of Fumarate and Tissue Extract on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

200 mg. of rat liver homogenate (unfasted rat), 0.5 ml. of 0.1 M K$_2$HPO$_4$, pH 7.4, 0.5 ml. of 0.0125 M fumarate, 1.0 ml. of rat muscle extract, 14.8 γ of dye in 0.1 ml. of ethanol, and water to a final volume of 3.0 ml. Incubated for 30 minutes.

Factors added			Over-all destruction of dye
Homogenate	Muscle extract	Fumarate	
			per cent
+	−	−	39
+	+	−	54
+	−	+	63
+	+	+	89
−	+	+	3

Results

In preliminary experiments only slight or no destruction of 4-dimethylaminoazobenzene was obtained even with 400 mg. of unfortified liver homogenate. By observing cold temperature precautions, the activity of the homogenate was improved. The addition of 1.0 ml. of 4:1 muscle extract (10) and 0.002 M fumarate enables much less homogenate to destroy a considerable quantity of dye (Table I). The requirements of the system for optimum ability to destroy dye were then studied.

The data in Table II demonstrate that DPN is essential for the destruction of dye, although the rate of destruction falls off rapidly with the coenzyme alone. The addition of 0.2 ml. of 0.6 M nicotinamide optimally decreased or eliminated the requirement for added DPN. However, as a precaution against variation in the preservation of the DPN in the homogenates, 1.0 mg. of DPN assaying 65 per cent, or its equivalent, was always added. No destruction of azo dye could be demonstrated in three

experiments when reduced DPN was employed in the absence of liver homogenate. For example, 790 γ of DPN (90 per cent reduced), prepared according to Green and Dewan (11), failed to react with 21 γ of 4-dimethylaminoazobenzene when incubated with or without nicotinamide, $MgCl_2$, and isotonic KCl buffered at pH 7.4 with KH_2PO_4.

TABLE II

Effect of DPN, Nicotinamide, and ATP on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 26 3 γ of dye and 0 4 ml. of homogenate (unfasted rat) per 3.0 ml. of reaction mixture incubated for 20 minutes. The molarity of the factor is the final concentration in the reaction mixture.

Factors added			Over all destruction of dye
DPN, 0 00023 M	Nicotinamide, 0 04 M	ATP, 0 0003 M	
			per cent
−	−	−	4
+	−	−	35
+	+	−	63
+	+	+	60
+	−	+	37
−	−	+	13
−	+	−	58
−	+	+	57

TABLE III

Effect of Oxidizable Substrates on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 45 5 γ of dye and 0 3 ml of homogenate (fasted rat) incubated for 20 minutes. The molarity is the final concentration in the reaction mixture.

Substrate added (0 004 M)	Over-all destruction of dye
	per cent
None	5
Hexose diphosphate .	47
Succinate . .	30
Malate . . .	34

A greater destruction of dye occurred when an oxidizable substrate was added. Malate, fumarate, succinate, α-ketoglutarate, and hexose diphosphate, each at a final concentration of 0.004 M, increased the over-all destruction of dye. In experiments comparing hexose diphosphate, malate, and succinate, the best response was obtained with hexose diphosphate (Table III). Oxalacetate occasionally stimulated the rate of destruction,

but its effect was variable. No dye was destroyed when succinate, malate, or hexose diphosphate was added in the absence of DPN and nicotinamide.

Magnesium ions were necessary for maximum activity. A final concentration of 0.003 M $MgCl_2$ increased the dye destruction by 25 per cent.

A requirement for adenosine triphosphate (ATP)[1] could not be demonstrated in the presence of DPN and nicotinamide (Table II), and only a slight stimulation occasionally occurred when ATP was added alone. If

TABLE IV

Effect of Various Enzyme Inhibitors on Destruction of 4-Dimethylaminoazobenzene by Rat Liver Homogenates

Systems as under "Methods" with 28.6 γ of dye and 0.4 ml. of homogenate (unfasted rat) incubated for 20 minutes. The molarity of the inhibitor is the final concentration in the reaction mixture. ATP at a final concentration of 0.0003 M was present in the mixtures under Experiment 3.

Experiment No.	Inhibitor added	Concentration	Over-all destruction of dye
		M	*per cent*
1	None		48
	Potassium iodoacetate	0.001	42
	Sodium azide	0.01	29
	p-Aminophenol	0.001	32
	Potassium fluoride	0.01	48
2	None		67
	Potassium malonate	0.01	63
	Hydroxylamine	0.01	43
	Sodium cyanide	0.01	27
3	None		55
	Guanidinoacetic acid	*0.003*	*55*
	DL-Methionine	0.003	55
	Potassium benzoate	0.003	53
	Atabrine	0.001	37

high energy phosphate is involved in this system, the needs are apparently supplied by the tissue and added reactants.

Heating the liver homogenates for 1 minute in a boiling water bath completely inactivated the system. The effect of adding various enzyme inhibitors to the system is presented in Table IV. Definite inhibition was produced by iodoacetate, *p*-aminophenol, hydroxylamine, cyanide, azide, and atabrine. Fluoride, semicarbazide, and malonate were without effect at the concentrations tried. The addition of DL-methionine, benzoate, or guanidinoacetic acid in the presence of ATP also did not alter the reaction rate or the final distribution of the metabolites of the dye.

During the reaction appreciable quantities of 4-monomethylaminoazobenzene, 4-aminoazobenzene, and 4'-hydroxy-4-dimethylaminoazobenzene

were formed from the added 4-dimethylaminoazobenzene. Generally the levels of the latter two metabolites increased with the length of the incubation period, whereas 4-monomethylaminoazobenzene was frequently present

TABLE V

Distribution of Azo Metabolites of 4-Dimethylaminoazobenzene in Fortified Rat Liver Homogenates Incubated with This Dye

Systems as under "Methods" with 0.4 ml. of homogenate (unfasted rat). The figures are micrograms of dye in 3.0 ml. of the reaction mixture.

Added dye or its metabolite*	DPN and nicotinamide omitted				Nicotinamide omitted				Whole system			
	0 min.	10 min.	20 min.	30 min.	0 min.	10 min.	20 min.	30 min.	0 min.	10 min.	20 min.	30 min.
DAB	27.8	26.8	26.0	26.4	27.8	20.2	19.2	20.1	27.8	12.0	4.1	1.3
MAB		0.6	1.2	1.1		1.1	1.4	1.6		1.3	0.6	0.3
AB			0.2	0.2		0.4	0.4	0.5		0.7	1.1	1.0
4'-HO-DAB						1.2	1.2	1.1		2.4	2.6	2.9

* DAB = 4-dimethylaminoazobenzene, MAB = 4-monomethylaminoazobenzene, and AB = 4-aminoazobenzene.
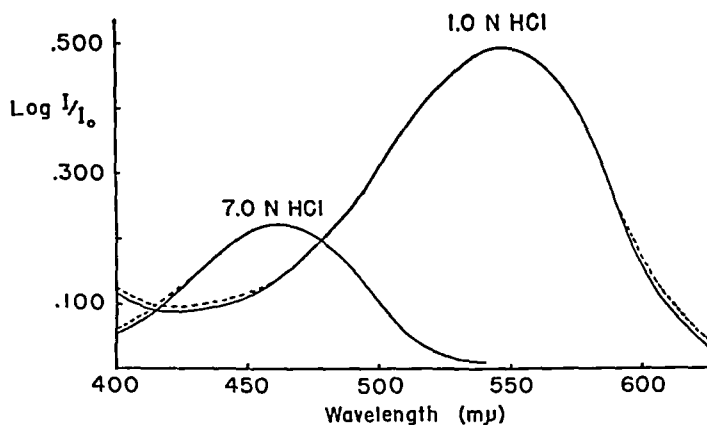

FIG. 1. The absorption spectra of 4'-hydroxy-4-dimethylaminoazobenzene (solid lines) and the acidic metabolite of 4-dimethylaminoazobenzene (broken lines) in 1.0 N and 7.0 N HCl. The concentration of the authentic dye was 7.5 γ and 1.11 γ per ml. respectively. Cell thickness = 1 cm. The curves were drawn so that the densities at 545 mμ (1.0 N HCl) and 465 mμ (7.0 N HCl) coincided.

in highest concentration after the first 5 to 10 minutes (Table V). Traces of a dye similar in its adsorption properties on Super-Cel to those of 4'-hydroxy-4-monomethylaminoazobenzene were frequently observed; however, no evidence for the presence of any 4'-hydroxy-4-aminoazobenzene was found.

The identities of the two basic dyes formed from the added dye were es-

tablished by their absorption spectra in acid and by mixed chromatograms with the known dyes, as previously described (1).

The identity of the acidic dye from the reaction mixture with 4'-hydroxy-4-dimethylaminoazobenzene was established in several ways. First, the absorption spectra of the metabolite in strong and weak acid corresponded closely with the spectra of the authentic compound (Fig. 1). Second, mixed chromatograms of the reaction product with the known dye gave only
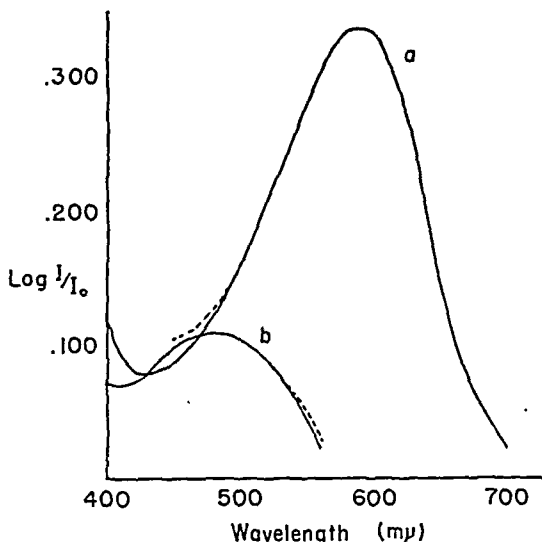

FIG. 2. The absorption spectra of the Schiff bases of the aromatic amines resulting om the reductive cleavage of 4'-hydroxy-4-dimethylaminoazobenzene (solid lines) and the acidic metabolite of 4-dimethylaminoazobenzene (broken lines). Curves a = Schiff bases of N,N-dimethyl-p-phenylenediamine in benzene; Curves b = Schiff bases of p-aminophenol in amyl acetate. Cell thickness = 1 cm. The curves were drawn so that the densities at 590 mμ (Curves a) and 485 mμ (Curves b) coincided.

a single zone with no evidence of resolution. Finally, the metabolite was reductively cleaved with $Na_2S_2O_4$ in acid solution and the amines formed were allowed to react with sodium β-naphthoquinone sulfonate at pH 7 (3). Two Schiff bases were formed, one extractable by benzene and the other subsequently extractable with amyl acetate. These properties and the absorption spectra of the bases (Fig. 2) in these solvents demonstrated that the amines were N,N-dimethyl-p-phenylenediamine and p-aminophenol, respectively. Approximately equimolar amounts of these amines were produced upon reduction of the acidic dye.

DISCUSSION

The enzymatic nature of the destruction of 4-dimethylaminoazobenzene by fortified rat liver homogenates is indicated by the observation that the system is inactivated by heat and requires the presence of DPN for activity.

The need of added substrate for maximum activity and the inability of reduced DPN to reduce the dye directly are further evidence that the reactions are catalyzed enzymatically. It is of interest that the livers of rats fed the dye contain less DPN than do livers of normal rats (12), and that the enzymatic destruction of testosterone (13) and α-estradiol (14) by rat liver minces also require DPN.

Presumably the reactions involved in the stepwise demethylation of 4-dimethylaminoazobenzene *in vivo* (1) are identical with those concerned *in vitro*. These may be oxidative demethylations similar to that involved in the enzymatic degradation of N-methylglycine to glycine and formaldehyde by liver mince (15). The stepwise oxidative demethylation of 4-dimethylaminoazobenzene occurs slowly in autoxidizing linoleic acid (16), but it is unknown to what extent a reaction of this type could account for the demethylations occurring in the liver homogenates. If it does occur, it must be catalyzed enzymatically. Transfer of the methyl groups seems unlikely, since the ability to donate methyl groups in the body is possessed by only a few of the naturally occurring methylated substances (17). Furthermore, it has not been possible to alter the carcinogenicity of either 4-dimethylaminoazobenzene or 4-monomethylaminoazobenzene by diets high in methyl donors or acceptors (18–20).

Since riboflavin is a potent inhibitor of the carcinogenic action of 4-dimethylaminoazobenzene (20–22), it is of interest that Kensler (23) recently reported that liver slices from rats fed high levels of this vitamin destroy the dye faster than liver slices from rats deficient in this factor. These facts suggest the possibility that demethylase, a riboflavin enzyme which oxidatively demethylates certain N-methyl-L-amino acids (24), may be involved in the demethylation of the azo dye in the rat liver. Similarly, the riboflavin enzyme quinine oxidase (25) which hydroxylates quinine might be involved in the hydroxylation of the azo dye.

No resolution of the demethylation and hydroxylation reactions in the liver homogenates has been achieved so far. It is apparent that a large fraction of the added dye that disappeared in these reactions was not recovered in the form of the several azo dyes tested for; this decrease may well result from the reductive cleavage of the azo linkages in these dyes. These reductions occur readily *in vivo* (3, 4).

While it is unknown to what extent the enzymatic hydroxylation of 4-dimethyaminoazobenzene in the 4' position proceeds *in vivo*, this reaction may be of significance, since the hydroxy dye is non-carcinogenic (9). This is in contrast to the stepwise demethylation of the dye in which the first demethylated product is as carcinogenic as the parent dye (18). Thus all of the available evidence still points to an azo dye very closely related to 4-dimethylaminoazobenzene as being the primary carcinogen when the dye is fed (2, 9).

SUMMARY

Methods are described by which the hepatic carcinogen 4-dimethyl-aminoazobenzene, its demethylated derivatives 4-monomethylaminoazobenzene and 4-aminoazobenzene, and the 4'-hydroxy derivatives of these three dyes can be determined in tissue extracts. With the aid of these methods rat liver homogenates fortified with diphosphopyridine nucleotide, nicotinamide, magnesium ion, and hexose diphosphate have been found to demethylate added 4-dimethylaminoazobenzene and to hydroxylate this dye to form a new metabolite, 4'-hydroxy-4-dimethylaminoazobenzene. More dye disappeared in these reaction mixtures than could be accounted for by the azo metabolites found.

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THE ESTIMATION OF MUCIN IN GASTRIC JUICE*

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The importance of mucin as a constituent of gastric mucus and of acid gastric juice has been generally recognized (2). Numerous methods for the quantitative determination of mucin have been described, the estimation of the reducing power having been the method almost universally used. The viscosity, acid-combining power, nitrogen content, and iodine-combining power have each been considered as a measure of mucin concentration; methods based on these properties have been applied to the whole gastric secretion or its contents or to various fractions isolated by ultrafiltration, electrodialysis, and the use of various protein precipitants (3–8). However, none of these methods has gained general acceptance (2).

Mucoitinsulfuric acid is regarded as a characteristic prosthetic group of gastric mucin and many other mucoproteins (9). It has been isolated from hog gastric mucus (10–13) and from pure canine gastric juice (14). Hexuronic acid (12) or, more specifically, glucuronic acid (9, 13) has been established as one of the four components of this polysaccharide. Since (gluc)uronic acid appears to be the most characteristic component of gastric mucin and several reliable methods for its estimation are available, we investigated the possibility of utilizing the estimation of glucuronic acid as a measure of the concentration of mucin in gastric juice. We have found that a modification of Tollens' naphthoresorcinol reaction for glucuronic acid (15) results in a consistent recovery of uronic acid from gastric mucin, mucoitinsulfuric acid, and whole canine gastric juice or mucus.

EXPERIMENTAL

The conclusions and opinions expressed in these studies are based on the results of more than 2800 determinations made in experiments of various types over a period of more than 2 years. The experiments presented here in the form of various tables and figures were selected on the basis of their illustrative value.

Preliminary Experiments

In our preliminary experiments we used the procedure outlined by Maughan, Evelyn, and Browne (16) for the determination of glucuronic

acid derivatives in urine as being the least complicated quantitative procedure of the reported modifications of Tollens' naphthoresorcinol reaction. When applied to gastric mucin, mucoitinsulfuric acid, and pure canine gastric juice, it produced a color characterized by the maximal absorption of light transmitted by Rubicon Filter 565. It is well known, however, particularly from the studies of Levene and his associates (9), that rather prolonged hydrolysis with strong acid is required for complete degradation of mucoitinsulfuric acids. Therefore it was necessary to establish conditions of hydrolysis which would result in the optimal recovery of uronic acid from mucin. Boiling for varying lengths of time with approximately 3 N hydrochloric acid in the presence of naphthoresorcinol was used by many investigators for the colorimetric estimation of uronic acids and their various derivatives (17–19). Similarly, boiling with comparable concentrations of hydrochloric acid was found to be successful in effecting hydrolysis of mucoitinsulfuric acid and chondroitinsulfuric acid with the liberation of their monosaccharide components. Accordingly, it was desirable to establish the optimal conditions for the hydrolysis of mucin compatible with the optimal development of color with naphthoresorcinol.

The sources of various preparations employed in this study were as follows:

The glucuronic acid was obtained from the A. D. Mackay Company, New York, and had a warranted purity of less than 5 per cent lactose and a melting point of 146° (uncorrected).

The menthylglucuronic acid was isolated from the urine of menthol-fed rabbits by the method of Williams (20). After the material had been purified by crystallizing it thrice, the melting point was 92.1° (uncorrected).

The gastric mucin was a preparation previously described by one of us (14). It was isolated from 15.4 liters of pure gastric juice obtained from dogs equipped with a gastric fistula and esophagotomy. The ash content was 0.50 per cent, and the elementary composition calculated for the ash-free substance was C 52.68 per cent, H 7.00 per cent, N (Dumas) 14.02 per cent, S (in the form of ethereal sulfate) 0.372 per cent, and P 0.00 per cent. The reducing power (Hagedorn-Jensen method after hydrolysis with N HCl for 6 hours at 100° in a sealed tube) was equivalent to 14.67 per cent glucose. The barium salt of mucoitinsulfuric acid was isolated in a high state of purity in a yield of 4.32 per cent. In all the experiments the preparation of gastric mucin mentioned above was dissolved in 0.02 N sodium hydroxide.

The mucoitinsulfuric acid (acid sodium salt) was isolated from Wilson's "gastric mucin" by the method of Levene and López-Suárez (11) and had an ash content of 4.97 per cent. The elementary composition (calculated

for the ash-free substance) was C 41.23 per cent, H 6.20 per cent, N (Dumas) 5.31 per cent, S (in the form of ethereal sulfate) 1.65 per cent, and P 0.00 per cent. The reducing power (Hagedorn-Jensen method after hydrolysis with N HCl for 6 hours at 100° in a sealed tube) was equivalent to 61.8 per cent of glucose. While this preparation was not of a high degree of purity, it compared favorably with those described by Levene (9). Aqueous solutions of this substance were used in all the experiments.

First we studied the effect of extended boiling without otherwise modifying the procedure of Maughan et al. Some of these results are illustrated in Fig. 1 (section A). If the boiling was continued for $4\frac{1}{2}$ hours, the optical density of the chromogen when measured with Filter 565 increased in a rather regular manner in the case of all the substances investigated, but there was a definite lag in the color development in the case of mucoitin-sulfuric acid as compared with glucuronic acid. That there was a relative lag also with mucin is evident from the fact that, while the optical density after 30 minutes of boiling was practically identical with that for glucuronic acid, there was a far greater development of color with mucin than with glucuronic acid or menthyl glucuronide. All curves tended to level off when boiling was continued for $3\frac{1}{2}$ to $4\frac{1}{2}$ hours. In a number of other experiments in which boiling was limited to $\frac{1}{2}$ to 1 hour, the lag in the color development with mucin was even more pronounced than in the experiments illustrated in Fig. 1. This phenomenon is unquestionably due to the fact that glucuronic acid as such is immediately available for the formation of chromogen, but when it is a constituent of mucin it must first be liberated in a free state. The behavior of menthyl glucuronide was very similar to that of glucuronic acid probably because it may be hydrolyzed with relative ease. The leveling off of the color development, observed with all the above substances when boiling is extended for $3\frac{1}{2}$ hours or more, can be explained by the fact that the chromogen formed with glucuronic acid and naphthoresorcinol under the conditions of our experiments reaches its maximum at about 4 hours. In this respect our observations confirm the earlier observations of Kapp (17) and Hanson et al. (19). Therefore it might be expected that with a sufficiently extended boiling time there should be no material difference in chromogen formation regardless of whether the glucuronic acid is available immediately as in the case of free glucuronic acid or whether it is only gradually liberated from mucin, provided the latter process is accomplished in a relatively short period of time.

The practical conclusions to be drawn from the above observations are that true recoveries of glucuronic acid may be expected with the procedure of Maughan et al. if boiling is extended to 4 hours or more, or that a shorter procedure might perhaps be developed if a certain degree of preliminary

hydrolysis of mucin preceded the "coupling" with naphthoresorcinol. In order to study the latter possibility, another series of experiments was carried out, in which the aforementioned substances were subjected to preliminary hydrolysis with 3 N HCl in a boiling water bath for 270 minutes, this being followed by "coupling" with naphthoresorcinol for a fixed period of 30 minutes. This procedure will be further referred to as "separate" hydrolysis and coupling, as distinct from the term "simultaneous" hydrolysis and coupling referred to in the experiments already described. The results of the "separate" hydrolysis and coupling experi-
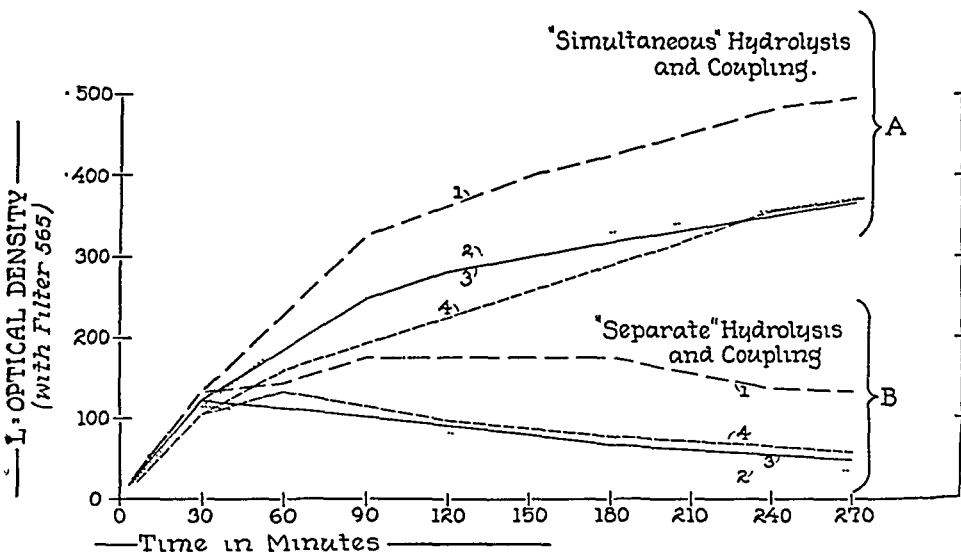

FIG. 1. Color development in the naphthoresorcinol reaction under various conditions of hydrolysis and coupling. A, Curve 1, gastric mucin (100 mg. per 100 ml.); Curve 2, glucuronic acid (1 mg. per 100 ml.); Curve 3, menthylglucuronic acid (2 mg per 100 ml.); Curve 4, mucoitinsulfuric acid (10 mg. per 100 ml). In B, the time includes 30 minutes coupling.

ments are illustrated in Fig. 1 (section B). A progressive fall in color intensity with glucuronic acid and menthyl glucuronide occurred in these experiments, as was to be expected in view of the well known fact that glucuronic acid is rather easily destroyed by boiling with strong hydrochloric acid. Similar relations occurred with mucoitinsulfuric acid, while the curve for mucin tended to rise till the end of 60 minutes and then to remain more or less constant.

When the recovery of glucuronic acid from mucin and mucoitinsulfuric acid was calculated from the light densities in each of the experiments graphically illustrated in Fig. 1, the following results were obtained. In

"simultaneous" hydrolysis and coupling experiments the recovery of glucuronic acid from mucin was considerably higher with 60 minutes than with 30 minutes boiling, and there was some tendency to a further increase if heating was extended further.   The recovery of uronic acid from mucoitinsulfuric acid after 30 minutes of "simultaneous" hydrolysis and coupling was not uniform, but after 60 minutes it was uniform and not materially increased when the time of hydrolysis was extended.   In experiments in which mucin and mucoitinsulfuric acid were subjected to preliminary "separate" hydrolysis, there was a steep increase in glucuronic acid recovery, especially from mucin.   However, this should be regarded as only an apparent effect, due to the more rapid destruction of free glucuronic acid in the standards than in uronic acid, which is gradually liberated from mucin.

TABLE I

Estimates of Glucuronic Acid Content (Per Cent) in Mucoitinsulfuric Acid and Mucin under Various Conditions of Hydrolysis and Coupling

	Simultaneous hydrolysis and coupling, Filter 565			30 min. hydrolysis followed by 30 min. coupling	
	30 min.	60 min.	240 min.	Filter 565	Filters 565 and 400
Mucoitinsul- furic acid	11.6 ± 0.6*	9.4 ± 0.4	6.94 ± 1.08	11.0 ± 0.2	12.8 ± 0.3
Mucin	1.08 ± 0.04	1.21 ± 0.06	1.63 ± 0.36	1.34 ± 0.04	1.28 ± 0.03

The above figures in each case represent the results of six determinations which were started simultaneously with the same solutions.
* Standard deviation = $\pm\sqrt{\Sigma d^2/(n-1)}$ (Fisher).

These experiments therefore indicated that extended "separate" hydrolysis could not well be employed because of the deterioration of the standards.   The highest recoveries of glucuronic acid from mucin and mucoitinsulfuric acid were obtained with the 30, 60, and 240 minute "simultaneous" procedure and also with the 30 minute preliminary "separate" hydrolysis. These procedures were subjected to more detailed study in order to determine more exactly the magnitude and also the reproducibility of the uronic acid recovery.

The results of a representative experiment are shown in Table I.   The highest mean recovery of glucuronic acid from mucin was obtained with the "simultaneous" procedure of 4 hours duration, but the recoveries were not consistent (coefficient of variation 22 per cent).   The next highest recoveries which were at the same time coincident with the smallest deviations (coefficient of variation 4 per cent) occurred when the mucin solu-

tions were subjected to separate hydrolysis for 30 minutes prior to the 30 minutes "coupling."

This method, as will be demonstrated below, produced reliable results when applied to gastric mucin and pure canine gastric juice, but was found to be less satisfactory when applied to alkaline or neutral mucus. The hydrolysis of alkaline or neutral mucus with hydrochloric acid gave rise to large amounts of furfural, which interfered with the development of color with naphthoresorcinol. However, furfural and glucuronic acid produce entirely different colors, as may be seen from the absorption spectra (Fig. 5). Maximum absorption in the visible spectrum in the case of furfural takes place in the range of light transmitted by Rubicon Filter 400 and in the case of glucuronic acid in the band transmitted by Filter 565. Straight line calibration curves were obtained for the light densities measured at these two wave-lengths for both of these substances. Therefore conditions are present which permit corrections for extraneous furfural by application of the principles suggested by Knudson et al. (21) for a two-component color system. With calculations based on this principle, described below under "Method," more satisfactory recoveries were obtained from alkaline mucus and from gastric juice containing considerable proportions of mucus.

Method

Reagents—

1. Hydrochloric acid. Reagent grade, concentrated, sp. gr. 1.19.

2. Naphthoresorcinol.[1] 0.2 per cent filtered (No. 42 Whatman filter paper) aqueous solution; must be prepared immediately before analysis.

3. Ether. Merck, reagent, treated with 1 per cent ferrous sulfate to remove peroxides, washed with water until sulfate-free, and stored over anhydrous sodium sulfate.

4. Ethyl alcohol. 95 per cent.

5. Standard solutions of menthyl glucuronic acid and glucuronic acid. A stock standard solution of menthyl glucuronic acid is prepared so as to contain 4 mg. per ml., which may be kept in the refrigerator for not more than a month. A dilute standard solution (1:100) is made up at the time of analysis. The standard solution of glucuronic acid, containing 0.02 mg. per ml., must be prepared immediately before analysis.

Procedure

*Hydrolysis—*A sample of material (gastric juice or solution of mucin), containing 0.01 to 0.6 mg. of uronic acid (2 ml. for histamine gastric juice,

[1] Naphthoresorcinol was obtained from the Schwarz Laboratories, Inc., 202 East 44th Street, New York 17, New York.

1 ml. for sham feeding juice, and 0.2 ml. for mucus), is pipetted into special calibrated colorimeter tubes,[2] and the volume is adjusted with distilled water to 2 ml.   Tubes with 2 ml. of a standard solution and 2 ml. of water (blank) are set up simultaneously.   1 ml. of concentrated hydrochloric acid is added, the contents being thoroughly mixed, and each tube is covered with a glass marble and placed in a boiling water bath for 30 minutes.

Coupling—The tubes are removed from the water bath, 2 ml. of naphthoresorcinol solution are added to each, and the tubes are thoroughly shaken.   1 ml. of concentrated hydrochloric acid is added.   The contents of the tubes are mixed and the tubes are covered and placed again in the boiling water bath for a further period of 30 minutes.

Chromogen Extraction—The tubes are withdrawn and cooled in an ice bath for 10 minutes.   2 ml. of ethyl alcohol are added to the contents and mixed, followed by 15 ml. of ether.   The tubes are stoppered with rubber stoppers[3] and shaken well by continuous, vigorous inversions for 30 seconds.   The contents of the tubes are allowed to settle for 10 minutes, and the upper purplish colored layer is read in a special tube holder in the Evelyn colorimeter, with Filter 565 for the single filter procedure and Filters 565 and 400 for the two-filter procedure, after the blank (reagent) tube has been set at 100.   The center setting should be no higher than 78. If it is any higher, the experiment must be discarded.   As a rule the excessive color is due to deterioration of the naphthoresorcinol.

Calculations—For the one-filter procedure, L_{565}^u/L_{565}^s = mg. of glucuronic acid per 100 ml. of material if 0.02 mg. of glucuronic acid is used as standard and 2 ml. of material are taken.

For the two-filter procedure,

$$\frac{K'_{565} \cdot L_{400}^u - K'_{400} \cdot L_{565}^u}{K'_{565} \cdot K_{400}^s - K'_{400} \cdot K_{565}^s} \times \frac{100}{V} = \text{mg. glucuronic acid per 100 ml. material}$$

where V = ml. of material taken, or

$$\frac{0.86 \cdot L_{400}^u - 2.70 \cdot L_{565}^u}{-11.8} \times 50 = \text{mg. glucuronic acid per 100 ml. material}$$

[2] Special tube holder, No. 4626, of the Rubicon Company, Philadelphia 32, Pennsylvania.

[3] These stoppers must be first thoroughly washed with acetone until the washings are colorless.   Before and after each set of determinations they are adequately rinsed with ether.   These stoppers are used for these determinations exclusively.   The suitability of any particular batch of stoppers is best indicated by the "center setting" of the blanks and the reproducibility of the calibration constants of the standard solutions.

if 2 ml. of material are taken and the calibration constants given below are used.

$$L^u{}_{400} = \text{optical density of unknown with Filter 400}$$
$$L^u{}_{565} = \text{``} \quad \text{``} \quad \text{``} \quad \text{``} \quad \text{``} \quad \text{``} \quad 565$$
$$L^e{}_{565} = \text{``} \quad \text{``} \quad \text{`` standard ``} \quad \text{``} \quad 565$$

Calibration Constants—K = (optical density)/(mg. per aliquot). With Filter 565, $K'_{565} = 0.86$ for furfural and $K^g_{565} = 4.72$ for glucuronic acid. With Filter 400, $K'_{400} = 2.70$ for furfural and $K^g_{400} = 1.09$ for glucuronic acid.

TABLE II

Reproducibility of Calibration Constants and Estimates of Glucuronic Acid Content in Various Substances with Proposed Procedures

Filter No.	Substance	n	Calibration constants*			Glucuronic acid		
			K	s.d.	Coefficient of variation	Per cent	s.d.	Coefficient of variation
565	Glucuronic acid	22	5.30	0.45	8.5			
	Menthylglucuronic acid	218	2.82	0.15	5.3	102.0†	4.5	4.4
	Mucoitinsulfuric acid	45	0.69	0.09	13.0	13.3	1.7	12.8
	Mucin	29	0.067	0.006	8.8	1.37	0.09	6.6
	Canine gastric juice	18‡	0.30	0.02	6.0	2.63	0.02	0.8
565 and 400	Mucoitinsulfuric acid	45				13.6	1.2	8.8
	Mucin	29				1.22	0.09	7.4

* K = (optical density)/(mg. or ml. per aliquot); s.d. = $\pm\sqrt{\Sigma d^2/(n-1)}$; coefficient of variation = (s.d./mean) × 100.

† From twenty-two determinations.

‡ Triplicate determinations of six dilutions of the same specimen, obtained after sham feeding from a dog with a gastric fistula and esophagotomy.

Since no preparations of gastric mucin of generally accepted purity, which could be employed as a standard, were described, the conversion of glucuronic acid values to those of mucin cannot be justified. However, in some of our studies (22), when such a conversion was deemed necessary for clarity of presentation, a conversion factor derived from the mean values of glucuronic acid content in our best preparation of mucin presented in Table II was used.

Absorption Spectra and Recovery Curves

Using the procedure described above, we have compared the absorption spectra of gastric mucin, mucoitinsulfuric acid, glucuronic acid, menthylglucuronic acid, furfural, and gastric juice of different degrees of purity.

From Fig. 2 it may be seen that the absorption curves for glucuronic acid and its derivatives and for pure canine gastric juice, such as that obtained, for example, at the height of the secretion produced by sham feeding, are identical, maximum absorption with Filter 565, while the curve for furfural (Fig. 5) is utterly different. These observations justify the use of single Filter 565 for pure gastric juice. The color development of the above reference substances and pure gastric juice follows Beer's law for optical densities measured with Filter 565 (Fig. 3). Straight line curves for glucuronic acid recovery for gastric mucin and mucoitinsulfuric acid were
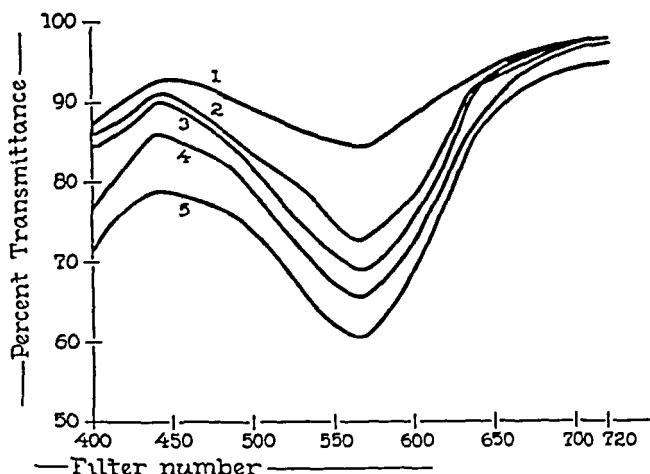

Fig. 2. Light absorption curves. Curve 1, pure gastric juice, sixth 15 minute fraction after sham feeding, pH 0.91, chlorides 162 milliequivalents per liter, pepsin 77 Mett units; Curve 2, barium salt of mucoitinsulfuric acid (10 mg. per 100 ml.); Curve 3, glucuronic acid (6 mg. per 100 ml.); Curve 4, gastric mucin (100 mg. per 100 ml.); Curve 5, menthylglucuronic acid (10 mg. per 100 ml.).

always obtained with the one-filter procedure, as illustrated by Fig. 4. The recovery of glucuronic acid in the experiments in which mucin was added to canine gastric juice was equally satisfactory.

Reliability of Method

The reproducibility of the one-filter procedure may be considered adequate in view of the values for standard deviations and coefficients of variation (Table II). In experiments performed over a period of 2 years in a routine manner with different batches of reagents and with a wide range of concentrations of all the substances studied, the coefficient of variation ranged from 5.6 to 12.8 per cent. Much greater uniformity was obtained in individual experiments even on a very large scale, as may be seen from Table I (last section), where the coefficients of variation for
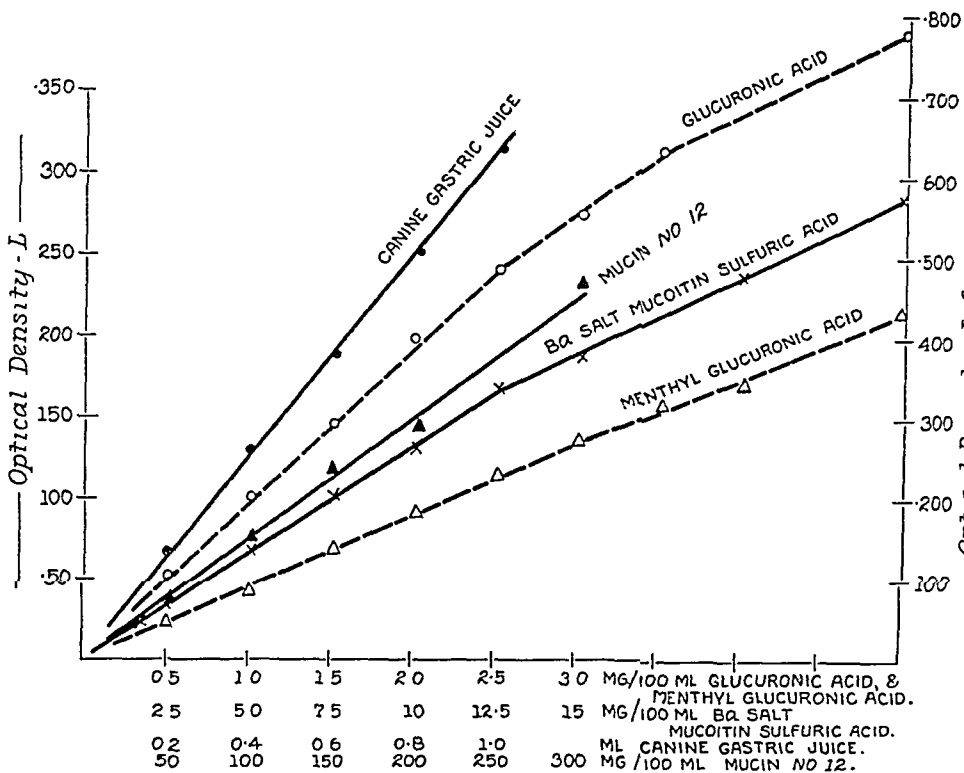
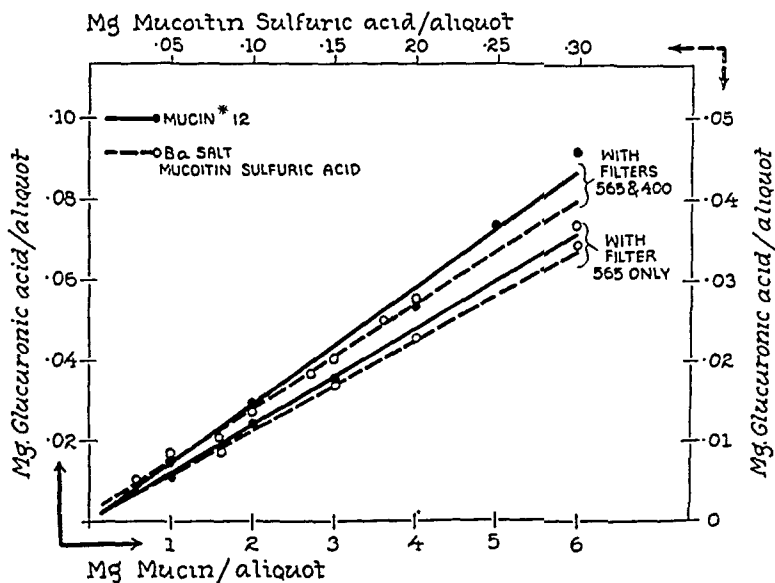Fig. 3. Calibration curves with one-filter procedure in terms of optical densities

Fig. 4. Calibration curves with two-filter procedure in terms of the estimated glucuronic acid content.

our routine procedure were only 1.8 per cent for mucoitinsulfuric acid and 3.0 per cent for mucin.

The absorption curves obtained with gastric juice which is not quite pure, particularly if mixed with mucus, have characteristics common to both glucuronic acid and furfural (Fig. 5). Many specimens of mucus (Fig. 5, Curve 5), especially those obtained from rats, and the first specimens of acid gastric juice collected in experiments on gastric fistula dogs (Fig. 5, Curve 4), which always contain a considerable amount of admixed mucus, give absorption curves closely resembling those of furfural. It was for such specimens that we found it necessary to use our two-filter

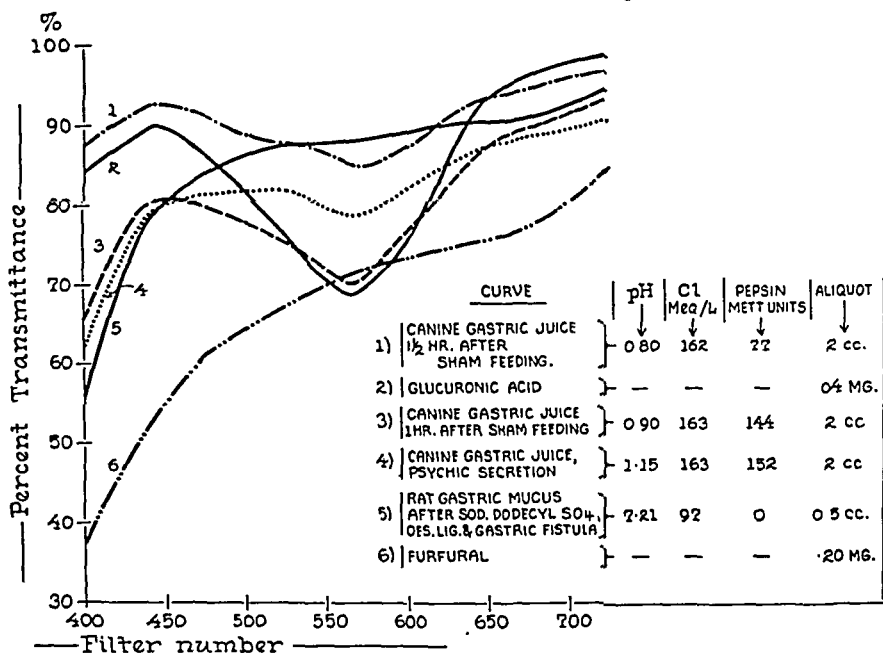| CURVE		pH	Cl Meq/L	PEPSIN METT UNITS	ALIQUOT
1)	CANINE GASTRIC JUICE 1½ HR. AFTER SHAM FEEDING.	0.80	162	77	2 CC.
2)	GLUCURONIC ACID	—	—	—	0.4 MG.
3)	CANINE GASTRIC JUICE 1 HR. AFTER SHAM FEEDING	0.90	163	144	2 CC
4)	CANINE GASTRIC JUICE, PSYCHIC SECRETION	1.15	163	152	2 CC
5)	RAT GASTRIC MUCUS AFTER SOD. DODECYL SO4, OES.LIG.& GASTRIC FISTULA	7.21	92	0	0.5 CC.
6)	FURFURAL	—	—	—	.20 MG.

FIG. 5. Comparison of the light absorption curves of various types of gastric secretion with those of glucuronic acid and furfural.

procedure. With mucin and mucoitinsulfuric acid, the reproducibility of the two-filter procedure was comparable to that obtained with the one-filter modification, as may be seen from the values for standard deviations and coefficients of variation in Table II. There was a slight difference in the magnitude of glucuronic acid recovery, but this does not seem to be significant. Straight line glucuronic acid recovery curves for mucin and mucoitinsulfuric acid resulted with both modifications (Fig. 4). Comparable recoveries with both modifications were obtained in experiments in which known amounts of mucin were added to canine gastric juice. However, the available evidence seems to indicate that truer recoveries

of mucin are obtained from alkaline gastric mucin and from not quite pure gastric juice with the two-filter than with the one-filter procedure. However, this evidence is not conclusive and further study is necessary.

Source of Uronic Acid in Gastric Juice

Mucoprotein should in all probability be regarded as practically the only source of the uronic acid that is liberated on acid hydrolysis of pure canine gastric juice. This is evident from the results of experiments in which uronic acid was determined in the filtrates after the removal of protein by several procedures; *viz.*, precipitation with acetone, basic lead acetate, and aluminum hydroxide. Acetone, under the experimental conditions obtaining, has been shown to precipitate all protein from freshly secreted canine gastric juice (23); precipitation with lead acetate at pH 6.4 to 6.8 has been generally regarded as one of the few specific precipitation procedures for mucoproteins, and we have found that aluminum hydroxide precipitates both the pepsin and the mucin of gastric juice quantitatively. Results obtained with gastric juice secreted in response to sham feeding are shown in Table III. Not more than 15 per cent, and in the majority of these experiments only 2 to 5 per cent, of the total glucuronic acid was recovered from these filtrates. Virtually all the glucuronic acid of the gastric juice (with a mean of 98.4 per cent) was recovered after the crystalloids had been removed by overnight dialysis. Similar results were obtained in several dialysis experiments with alkaline mucus collected from dogs with a gastric fistula and esophagotomy.

It is known that pepsin in an acid medium slowly digests mucoproteins, and peptic digestion has in fact been extensively used in the past as a preliminary step in isolating the carbohydrate complex of mucoproteins, especially chondroitinsulfuric acid. In our experiments with protein precipitants, the specimens of gastric juice were subjected to analysis not immediately after collection but after standing at 5° for 3 to 24 hours. Some degree of hydrolysis may therefore have taken place with the splitting off of mucoitinsulfuric acid and its derivatives, and this may account for the small amounts of glucuronic acid found in the deproteinized filtrates. Therefore it is justifiable to conclude that the uronic acid of the gastric juice is derived predominantly or perhaps even exclusively from its protein constituents.

Mucin Content of Gastric Secretion under Different Conditions of Stimulation

The method described above was instrumental in establishing several physiologically important facts concerning the quantitative aspects of mucin secretion in relation to the nature of the stimulation. This part of

the study will be published in detail elsewhere (24). However, several observations should be stressed here. Table IV shows that there were exceedingly wide variations in the mucin concentration of different types

TABLE III

Partition of Uronic Acid Derivatives in Canine Gastric Juice

Specimen No.	pH	Chloride	Pepsin, Mett units	Total glucuronic acid	Non-dialyzable fraction*	In filtrates* after precipitation with		
						Lead sub-acetate	Aluminum hydroxide	Acetone
		m.eq. per l.		*mg. per 100 ml.*				
1	0.78	160	85	2.90	89 (8)	5 (2)		
2	0.96	154	31	1.56		10 (2)	6 (4)	
3	0.91	167	92	1.44		2 (2)	2 (12)	8 (2)
4	0.90	164	98	1.35	99 (12)	9 (2)		10 (2)
5	0.98	165	117	0.98	115 (4)	5 (2)	5 (8)	15 (2)
6	0.94	171	41	0.68			7 (12)	
7	1.00	172	23	0.61		0 (2)	10 (2)	

* Expressed in percentage of total glucuronic acid.

The figures in parentheses represent the number of experiments with respective specimens.

Gastric juice or mucus was introduced into cellophane tubing (Nojax, Visking Corporation) and allowed to dialyze against tap water, distilled water, or physiological saline.

For precipitation with lead subacetate (Merck) the "free" acid in the gastric juice was neutralized with a calculated amount of 1.0 N NaOH, and 0.1 N NaOH was added to make the pH 6.8 to 7.0. 10 per cent lead subacetate was then added from a burette until no further precipitation was obtained. After standing in the refrigerator overnight, the sample was centrifuged and filtered. The pH of the filtrate ranged from 6.4 to 6.8.

For precipitation with colloidal aluminum hydroxide two procedures were used which gave comparable results. (1) The "free" acidity of the aliquot of gastric juice was neutralized with a calculated amount of N NaOH, and 0.1 to 0.2 volume of colloidal aluminum hydroxide was added. The solution was left in the refrigerator overnight and the supernatant was then filtered through No. 40 Whatman filter paper. (2) 0.25 volume of 10 per cent aluminum chloride was added to gastric juice and the necessary amount of NaOH to make the pH 5.8 to 6.9 (as previously determined in a separate sample) was added rapidly with vigorous mixing. After the solution had stood overnight in the refrigerator, the supernatant was filtered through No. 40 Whatman filter paper.

Precipitation with acetone was carried out as previously described (23).

of gastric secretion which were equivalent to 0.06 to 28.6 mg. per cent of glucuronic acid. The lowest concentrations were found in the secretion following histamine administration, the highest in pure alkaline mucus secreted by the stomach of a fasting animal, while those for gastric juice

obtained after sham feeding were in the middle range. These wide variations are important methodologically, since (a) they should be considered in selecting the size of the aliquots to be taken for mucin determination, and (b) they indicate that the magnitude of error inherent in the method described is sufficiently small not to jeopardize its value in physiological studies.

TABLE IV

Mucin Concentration (As Glucuronic Acid) in Gastric Secretion under Various Conditions of Stimulation

No. of experiments	Dog	Type of secretion	Rate	pH	Pepsin, Mett units	Glucuronic acid	
			ml. per hr. per kg.			*mg. per 100 ml.*	*mg. per hr. per kg.*
During	A	Fasting	0.53	8.02	0	14.1	0.07
same		Sham feeding	12.5	1.08	58	2.6*	0.32
experiment		" " and histamine	12.5	0.94	3	0.34*	0.04
5		Spontaneous mucus	0.52	8.20	0	7.56	0.04
4		Sham feeding, total secretion	9.3	1.03	33	1.84*	0.18
1		Sham feeding, height of secretion	10.2	0.93	31	0.61*	0.06
2		After histamine	14.0	0.92	<1	0.06	<0.01
5	B	Sham feeding, total secretion	>8.8	0.92	85	1.50*	>0.13
2		Spontaneous mucus	0.26	7.80	0	6.75	0.02
1		+ atropine	0.14	8.30	0	28.6	0.04
2		After sodium dodecyl sulfate	4.6	7.70	0	13.4	0.62
1		+ atropine	6.6	8.70	0	23.5	1.55

* Mucin in solution.

DISCUSSION

Glucuronic acid is regarded as a component of mucoitinsulfuric acid, the characteristic prosthetic group of gastric mucin (9, 13). Consequently we selected glucuronic acid or menthyl glucuronide or both as reference substances and expressed the results of our analyses in terms of glucuronic acid. The absorption curves obtained by us for glucuronic acid or its derivatives were quite different from those which we obtained for furfural. This provides further confirmation of the view, first expressed by Mandel

and Neuberg (25), that furfural is not responsible for the characteristic with HCl, as has been postulated by Tollens and many other investigators. The absorption curves produced by mucin and by pure gastric juice were strikingly similar to the curves produced by glucuronic acid, while those for alkaline gastric mucus showed features common to the curves for both glucuronic acid and furfural. This indicates that any method based on the determination of furfural alone cannot be utilized for the estimation of mucin. Furthermore, furfural formed from sources other than mucin was found to be detrimental to the estimation of mucoproteins by the naphthoresorcinol method if the calculations were based solely on the light density read at the band of maximum absorption with Filter 565. Only correcting for extraneous furfural made possible by application of the two-filter procedure seemed to make the naphthoresorcinol method more specific and to result in satisfactory recoveries from pure and not too heavily contaminated gastric juice.

We are aware that the method proposed here for the estimation of mucin in gastric juice does not meet the most exacting requirements of quantitative analysis, but to our knowledge it is the only method described so far which has been subjected to an exhaustive series of tests of reliability and has given results reproducible within 10 per cent. We believe that this method will be valuable in the solution of many important problems in the physiology and pathology of the gastric glands.

SUMMARY

A quantitative method for the estimation of mucin in the gastric juice and gastric contents has been developed, based upon the determination of glucuronic acid, a characteristic component of the prosthetic group of mucoproteins. The uronic acid is determined by a photoelectric-colorimetric method by the use of Tollens' naphthoresorcinol reaction, as modified by Maughan, Evelyn, and Browne (16), after preliminary acid hydrolysis of the material. With gastric mucin and its derivatives the resulting color is a two-component color system with two maxima of light absorption obtained with Filters 565 and 400. The former band is characteristic for uronic acid itself and the latter for furfural, which may be derived either from uronic acid or from other substances, as in the case of gastric mucus or not quite pure gastric juice.

Reproducible results were obtained for mucin, mucoitinsulfuric acid, and pure gastric juice from the light densities determined in an Evelyn photoelectric colorimeter with Filter 565 alone, glucuronic acid or menthyl glucuronide being used as a standard. For mucus and contaminated gastric juice, it was necessary to determine light densities with Filters 565 and 400 by calculations based on the principles developed by Knudson,

Meloche, and Juday (21). This procedure gave reproducible results also with mucin and mucoitinsulfuric acid. Fractionation experiments with various protein precipitants and dialysis demonstrated that only insignificant amounts of uronic acid were present in the protein-free fractions of canine gastric juice. It is probable that these small quantities may be derived from products of the enzymatic hydrolysis of mucin.

The concentration of mucin varied greatly in different types of gastric secretion. It was highest in alkaline mucus secreted either spontaneously or in response to intragastric instillation of sodium dodecyl sulfate and lowest in gastric secretion provoked by histamine administration. The concentration of mucin in the juice secreted in response to sham feeding was much higher than that of the gastric juice following histamine administration.

Addendum—Recently, after this study had been virtually completed, a new and specific color reaction for hexuronic acid with carbazole was reported by Dische (26), who claimed it to be suitable for the quantitative determination of hexuronic acid in various uronides and possibly also in some biological fluids. However, the presence of excessive amounts of protein in proportion to uronic acid appeared to jeopardize the results. We have attempted to explore the possibility of the application of this reaction to the determination of mucin in gastric secretion. The color development with glucuronic acid and menthylglucuronic acid (1 and 2 mg. per cent), when measured by the Evelyn colorimeter with Rubicon light Filter 520, was reproducible with a 12 per cent coefficient of variation, and the recovery of glucuronic acid from menthyl glucuronide was within 1 per cent of the theoretical value. The absorption spectra produced with our preparations of mucoitinsulfuric acid and gastric mucin were identical with that of glucuronic acid. A straight line recovery curve for hexuronic acid was obtained in experiments with gastric mucin (in a range of 100 to 400 mg. per cent) and mucoitinsulfuric acid (in a range of 3.0 to 50 mg. per cent) solutions. The uronic acid content, determined by the carbazole method, was 1.55 ± 0.08 per cent for mucin and 11.1 ± 1.1 per cent for mucoitinsulfuric acid. The reproducibility of the carbazole method therefore approximates that of our naphthoresorcinol method. In view of the greater simplicity of the carbazole method, and especially in view of its specificity, it deserves to be explored further with the purpose of applying it to the estimation of mucin in gastric secretion and the gastric contents.

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roughly resembled that of vertebrate rhodopsin and the visibility spectrum of the living cephalopod eye (11).

The fact that the squid contains a rich store of a photostable or nearly photostable "visual purple" is of interest in view of the long accepted dictum that stimulation of the retina by light is invariably accompanied by the bleaching of a visual pigment. Hartline, Milne, and Wagman (12) have recently reported an interesting physiological study of the retina of the horseshoe crab *Limulus*, which like the squid contains a formaldehyde-sensitizable pigment. It was found that the number of quanta absorbed by the visual pigment (13) in bringing about a constant electric response of the eye was not constant but was greater when the sensitivity of the eye was decreased by light adaptation. This suggests that the amount of unbleached visual pigment was greater than that necessary to account for the decreased sensitivity of the light-adapted eye. Thus the bleaching of the visual pigment of *Limulus* appears insufficient to explain the decreased sensitivity of the light-adapted eye. Nevertheless, the kinetics of light and dark adaptation of the *Limulus* eye appear to be otherwise identical with those of vertebrate eyes (14).

It is possible, therefore, that a more detailed study of these curious invertebrate retinal pigments may prove important to the photochemistry of vertebrate as well as invertebrate vision. The following questions are considered in this paper: (1) What is the true absorption spectrum of the formalin-sensitizable pigment of the squid, in the absence of the large quantities of melanin present in earlier extracts? (2) Are the intermediate pigments of the vertebrate visual cycle, such as indicator yellow, formed when the squid "visual purple" is bleached?

EXPERIMENTAL

Preparation of Melanin-Free Retinal Extracts—Vertebrate visual purple can be extracted from the retinal rod cells by mild detergents such as aqueous digitonin. However, such extracts may contain large amounts of light-absorbing impurities such as proteins, melanin, and oil droplets. These impurities can be greatly reduced by peeling off the melanotic screening layer either mechanically or by Saito's method: centrifugation of retinal suspensions in concentrated sucrose (15). In the latter case, the retinal rods centrifuge upwards and the melanin downward. Hardening the retina in alum (4 per cent potassium aluminum sulfate) or, less drastically, in a buffer at about pH 4.5 is also of great value in reducing the solubility of both proteins and melanin. Oil droplets are removed by washing with non-polar solvents such as petroleum ether.

Unfortunately, the melanin of the squid retina, although forming a distinct posterior screening layer, is partly within the retinal rod layer

and therefore cannot be peeled off. However, a nearly melanin-free, bright red layer could be obtained by blotting the anterior layer on filter paper. The red pigment was rapidly bleached by dilute formalin in the light, or by chloroform in the dark, and the bleaching was accompanied by the release of retinene. The effect of formalin was reversible, since rinsing the retinas in sea water abolished the light sensitivity.

An attempt was made to purify the red pigment by pretreatment of the retinas with alum, but destruction of the pigment resulted. However, a combination of Saito's method of sucrose homogenization and washing with an acid buffer proved successful and less painstaking than the mechanical separation described above. Squid retinas were dissected in ordinary daylight and preserved at $-20°$ until used. Two medium sized retinas were then ground about a minute in a Potter homogenizer with 5 ml. of a solution containing 2 gm. of sucrose and 3 ml. of water. Centrifugation yielded a black bottom layer and a red top layer, separated by opalescent red fluid. The top layer and red fluid were mixed, diluted with an equal volume of water, and centrifuged. The sediment was washed first with 0.1 M Na_2HPO_4 and then with cold phosphate-citrate buffer, pH 4.5. From this point on all operations were conducted as rapidly as possible in the cold. The red sediment was extracted for 2 minutes at $0°$ with 0.5 ml. of 3 per cent aqueous digitonin. Centrifugation yielded a clear red solution which was pipetted into a 2 mm. cell previously cooled with its holder in the freezing compartment of the refrigerator. The absorption spectrum was then measured with a potentiometric spectrophotometer (16). The spectrum, corrected for the absorption of the cell and solvent is shown by Curve 1 of Fig. 1. The absorption peak is at about 495 mμ, slightly but distinctly displaced toward the blue from that of vertebrate rhodopsin, which is 502 mμ. The absorption spectrum of frog rhodopsin, prepared from alum-pretreated retinas, was determined on the same instrument and is presented also in Fig. 1 (Curve 2).

Stability—The squid pigment was quite stable before extraction, and squid from local fish markets gave high yields, even though their retinas were thoroughly distintegrated by the time they were bought. However, the case was quite the opposite in aqueous digitonin, the absorption peak declining about 25 per cent in 30 minutes at pH 6.1 and 25.0°. The decomposition was not due to the light, since it was not accelerated by a 10 minutes exposure to a 100 watt light at 6 inches distance. 1 minute of such illumination brought about nearly complete bleaching of frog visual purple.

Photosensitivity of the squid "visual purple" was readily induced by addition of formaldehyde to make a concentration of 0.5 M. Since the thermal decomposition of the pigment was also increased, to about double

the normal rate, rapid measurement of the spectrum was obligatory. The effect of light on a formalized extract is shown in Fig. 2 and indicates a photosensitivity about 5 per cent that of vertebrate visual purple.

Squid "Indicator Yellow"—Photic or thermal decomposition of rhodopsin results in the liberation of indicator yellow, which in mildly acid buffers fades in the course of about 3 hours. It was of interest to determine
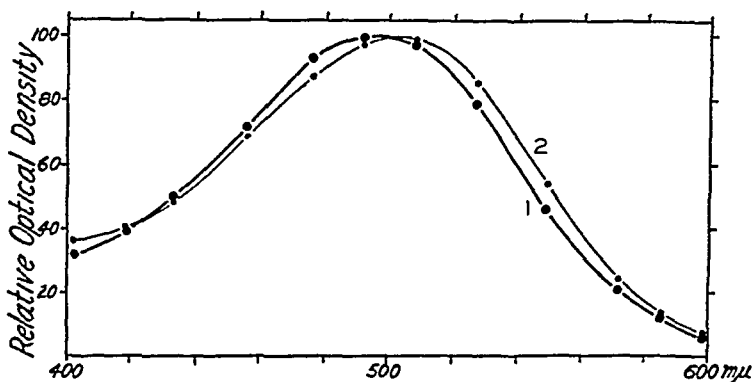

FIG. 1. Curve 1, absorption spectrum of purified "squid visual purple;" Curve 2, purified frog visual purple.
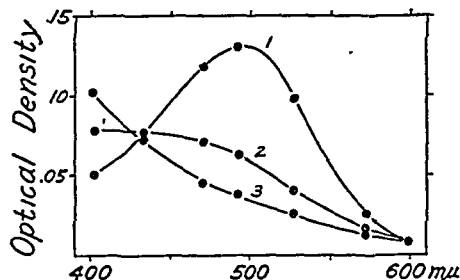

FIG. 2. Bleaching of "squid visual purple" by light in 0.5 M formaldehyde. Curve 1, before exposure to 100 watt light at 1 foot at 25°; Curve 2, after 10 minutes in light; Curve 3, after 20 minutes in light.

whether indicator yellow played a similar rôle as an intermediate in the decomposition of the photostable retinene precursor of the squid. The following procedure, used by Lythgoe for the demonstration of vertebrate indicator yellow, was followed. 0.01 ml. of 10 N NaOH was added to 0.5 ml. of an extract of squid "visual purple," causing bleaching of the pigment in a few seconds, as shown by Curve 2 of Fig. 3. After 2 hours at room temperature, 0.02 ml. of concentrated HCl was added. The extract quickly became deep yellow, due to the development of an absorption peak at 445 mμ. These changes are very similar to those shown by frog indicator

yellow. The absorption spectrum of the acid pigment was quite stable at room temperature, as reported by Lythgoe for acid indicator yellow below pH 4.

Pigments having indicator properties and soluble in petroleum ether containing polar solvents have been extracted from squid as well as from vertebrate retinas. In the case of the squid, two retinas were soaked in 0.1 M Na$_2$CO$_3$ for 15 minutes, ground with acetone, and extracted from 50 per cent acetone by petroleum ether. The residue obtained by drying the petroleum ether was colorless but became bright orange on addition of acid chloroform, due to an absorption peak at about 475 mμ.
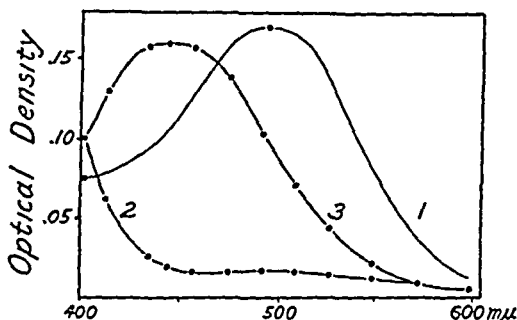

FIG. 3. Effect of pH on bleaching products of "squid visual purple." Curve 1, density at 402 and 495 mμ before bleaching; Curve 2, after adding NaOH; Curve 3, after adding HCl.

DISCUSSION

It is believed that the squid "visual purple" has been prepared in as pure a state as the best extracts of vertebrate visual purple, because the methods employed are known to reduce the solubility of protein and melanin impurities in such extracts to nearly negligible levels. Melanin (17) is particularly dangerous because of its high extinction coefficient. However, its solubility at pH 4.5 is so low that retinal material containing relatively enormous amounts of melanin yields extracts only slightly less pure than those prepared from demelanized homogenates.

A detailed comparison of the vertebrate and invertebrate "visual purples" may be of value in an attempt to assign a rôle in the visual cycle to the squid "visual purple." Such a comparison is presented in Table I.

The bleaching by light of the formalin-sensitized squid "visual purple" serves merely as a device whereby it is possible to demonstrate a latent photosensitivity of the red pigment. It should not be inferred that formaldehyde itself is present in the living retina, or that formaldehyde is

unique in this action. Furthermore, the most recent measurements (18) on living squid retinas show no effect of light on their retinene precursor. Because of this unusual light stability, a specific name, cephalopsin, has been proposed for the visual purple of the squid.

It thus appears that the view of the nineteenth century workers, that the squid "visual purple" was photochemically active, may have been closer to the mark than more recent workers may be willing to concede. However, it does not yet appear possible to frame a more specific conclusion than the following (10):

"Just how related all this is to the vision of the squid is hard to say. It may merely be that the normal squid photopigment is relatively light-stable, and that the formalin treatment renders it light-unstable. In that case serious consideration must be given to the possibility that the bleaching of vertebrate visual purple is a specialization and that the absence of bleaching, as in photosynthesis and photodynamic action, may have no direct bearing on the efficiency of a visual pigment."

TABLE I

Properties of Purified "Visual Purples"

	Frog	Squid
Absorption peak, $m\mu$	502	495
Thermal stability	High	Low
Photic "	Low	High
Photosensitizers	H_2O	H_2O + denaturants
Effect of polar solvents .	Bleaching	Bleaching
Intermediates of bleaching	Indicator yellow	Indicator yellow
Acid digitonin, $m\mu$	445	445
Basic "	Colorless	Colorless
End-product of bleaching	Retinene	Retinene

SUMMARY

1. The squid retina contains a relatively light-stable red pigment with maximum absorption at 495 mμ, which can be obtained in a nearly melanin-free state by centrifugation of retinal homogenates in 40 per cent sucrose. The pigment can be extracted from the non-melanoid fraction at pH 4.5 by aqueous digitonin.

2. The pigment is bleached by light in the presence of dilute denaturants or in the dark by higher concentrations, yielding a colorless product in alkaline solutions. The colorless product becomes deep yellow in acid solutions.

3. The bleaching process releases retinene.

4. All properties of the squid visual purple thus far investigated closely *resemble those of the vertebrate visual purples,* with the exception that

the squid pigment is relatively unstable thermally and is not bleached by light.

5. It is suggested that the light sensitivity of the normal squid photo-pigment may be independent of its light stability.

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STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, PLASMA COPPER, AND PLASMA IRON IN PROTEIN-DEFICIENT AND IRON-DEFICIENT SWINE*

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In a previous communication (1) values for free erythrocyte protoporphyrin, plasma copper, and plasma iron in normal and in pyridoxine-deficient swine were presented. The values in the normal group were found to be 118 ± 43.4, 206 ± 26.3, and 169 ± 38.8 γ per cent respectively. In the pyridoxine-deficient group on the other hand, the values were 47 ± 13.6, 160 ± 38.8, and 468 ± 166.6 γ per cent, respectively. Because of the small amount of free protoporphyrin in the erythrocytes of the pyridoxine-deficient animals, and because a fall in the erythrocyte protoporphyrin was noted long before the development of anemia in animals in which pyridoxine deficiency was induced, it was suggested that the fundamental disturbance in anemia due to pyridoxine deficiency may be a failure in protoporphyrin synthesis.

The purpose of this report is to present the results of similar determinations in swine with anemia due to protein deficiency, iron deficiency, and acute hemorrhage. In addition, values for the iron-binding capacity of the serum in normal, protein-deficient, and iron-deficient swine are given.

Materials and Methods

For this study thirty-nine weanling pigs were used. Twenty-four animals were placed on a low protein diet and fifteen were fed a diet low in iron but adequate in all other respects.

The basal diet of the low protein group consisted of either Sheffield "new process" casein 10.0 per cent, sucrose 73.8 per cent, lard 11.0 per cent, and swine Salt Mixture 3 (2) 5.2 per cent, or casein 10.0 per cent, sucrose 57.7 per cent, lard 27.1 per cent, salt mixture 5.2 per cent. The basal diet of the iron-deficient group consisted of casein 26.1 per cent, sucrose 57.7 per cent, lard 11.0 per cent, and swine Salt Mixture 3 (with iron pyrophosphate omitted) 5.2 per cent. In addition, all animals were given cod liver oil (Mead Johnson, 1800 units of vitamin A, 175 units of vitamin D per gm.) 0.5 gm. per kilo of body weight daily, or Natola (Parke, Davis, 55,000

units of vitamin A, 11,000 units of vitamin D per gm.) 0.056 gm. per kilo of body weight per week. Vitamins were supplied in crystalline form in capsules and were administered orally three times a week. The quantities of crystalline vitamins were, with the exceptions noted below, thiamine hydrochloride 0.25, riboflavin 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20, calcium pantothenate 0.50, choline chloride 10.0, p-aminobenzoic acid 0.10, inositol 0.10. All animals except Pigs 9-13 to 9-63, inclusive, received crystalline biotin 50 γ per kilo of body weight per week intramuscularly. Pigs 10-45 to 10-59, inclusive, received the high fat diet and 50.0 mg. of choline rather than 10.0 mg. Pigs 10-24 to 10-44, inclusive, were given no p-aminobenzoic acid or inositol. Pteroylglutamic acid, either 30 or 100 mg. per kilo of body weight daily, was added to the vitamin supplement of Pigs 10-24 to 10-58. Pigs 10-37, 10-40, 10-41, 10-42, 10-43, and 10-44 received no niacin in the vitamin supplements during the first part of the experiment but had been treated with adequate amounts of this vitamin for at least 30 days prior to the determinations included in the present report. Full details of the experimental methods have been published elsewhere (2).

Erythrocyte protoporphyrin determinations were made by the method of Grinstein and Watson (3). Plasma copper was measured by the method of Cartwright, Jones, and Wintrobe (4). For the determination of plasma iron the method of Kitzes, Elvehjem, and Schuette (5) as well as the method of Barkan and Walker (6) was used. A modification[1] of the method of Schade and Caroline (7) was used for the measurement of the iron-binding capacity of serum. Serum proteins were determined by the biuret method (8) with the modification of Kingsley (9). By this procedure the values for ten control animals (26 per cent casein) were found to be as follows: total serum protein 6.15 ± 0.437 gm. per cent, serum albumin 3.72 ± 0.173 gm. per cent, serum globulin 2.43 ± 0.223 gm. per cent.

Results

Protein Deficiency—The morphologic characteristics of the anemia observed in these animals are presented in Table I. The anemia was usually mild in degree. The mean value for the volume of packed red cells for twenty-two pigs was 35.7 ml. per 100 ml. The mean value for normal pigs is approximately 45.0 ml. per 100 ml. and values below 40 ml. are rarely encountered. The anemia was normocytic and normochromic and was not accompanied by reticulocytosis. Leucopenia and thrombocytopenia were not present.

Chemical findings are presented in Table II. In all the animals hypo-

[1] Cartwright, G. E., Black, P., and Wintrobe, M. M., *J. Clin. Invest*, in press.

proteinemia and especially hypoalbuminemia were present. The mean value for erythrocyte protoporphyrin was not significantly altered from the normal, being 124 ± 35.6 γ per 100 ml. of red blood cells. The mean

TABLE I

Morphologic Data for Protein-Deficient Swine

Pig No.	Age at beginning of experiment	Days on experiment	Weight	Red blood cells	Hb	Volume of packed red blood cells	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Reticulocytes	White blood cells	Platelets
	days		kg.	millions per c.mm.	gm. per cent	ml per 100 ml.	cu. micra	micro-micro-grams	per cent	per cent	thousands per c.mm.	thousands per c.mm.
9-61	31	134	22.7	5.75	10.4	31.5	55	18	33	0.4	12.6	566
9-62	21	154	18.6	5.72	11.5	34.5	60	20	33	2.8	19.3	570
9-63	21	112	6.4	4.13	6.5	21.0	51	16	31	2.8	6.2	354
10-24	70	183	49.8	7.40	12.7	39.0	53	17	33	0.4	17.7	400
10-25	60	183	54.5	7.83	13.8	41.0	52	18	34	0.8	27.4	490
10-27	70	183	44.0	6.94	12.7	39.0	56	18	33	1.5	17.5	300
10-29	60	183	65.6	6.75	13.8	39.0	58	20	35	0.6	17.0	430
10-33	70	183	19.5	7.21	12.1	38.0	53	17	32	4.8	21.8	400
10-34	60	183	79.7	7.35	12.9	41.0	54	17	31	0.5	24.1	380
10-35	70	183	56.4	7.01	11.9	35.2	50	17	34	0.9	15.4	380
10-37	21	206	31.7	6.22	11.7	36.0	58	19	32	2.2	13.9	430
10-40	21	206	17.0	6.30	10.9	35.0	55	17	31	1.4	13.6	350
10-41	21	206	39.1	7.10	13.8	40.6	57	19	34	1.8	11.5	330
10-42*	21	206	15.9	2.29	3.2	12.0	52	14	28	9.8	18.0	280
10-43†	21	180	21.6	4.27	7.8	24.0	56	18	32	0.2	17.7	380
10-44	21	206	30.7	6.33	11.7	37.0	59	19	32	3.0	20.4	340
10-45	27	156	13.7	6.96	12.0	36.0	52	17	33	1.0	15.1	440
10-46	27	156	17.7	6.36	11.8	35.2	55	19	33	2.6	17.8	480
10-47	27	156	20.5	5.31	8.9	27.0	51	16	33	2.0	13.7	490
10-48	27	156	10.7	6.35	12.0	36.4	57	19	33	1.0	14.5	460
10-50	27	156	19.3	6.51	11.3	34.4	53	17	33	0.6	12.3	410
10-57	30	137	20.4	5.87	12.3	37.2	63	21	33	0.4	10.9	400
10-58	30	137	22.5	6.19	12.1	35.6	58	20	34	0.4	14.0	430
10-59	30	137	17.6	6.45	11.7	35.4	55	18	33	1.2	17.2	450
Mean.	38	168	30.8	6.46	11.8	35.7	55	18	33	1.5	16.1	422

* Terminal hemorrhage; values not included in the mean.

† Infection; values not included in the mean.

value for plasma iron was 115 ± 30.1 γ per cent as compared wi[...]
38.8 for the normal group. This represents a significant lowering
iron, although not to the extent seen in iron-deficient [...]

stance were values as high as those seen in normal animals observed in protein-deficient pigs. The correlation between the degree of hypoproteinemia and the iron-binding capacity of the serum is shown in Fig. 1. With a reduction in the total serum proteins there was a proportional reduc-

TABLE II

Chemical Data for Protein-Deficient Swine

Pig No.	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum	Total serum proteins	Serum albumin	Serum globulin
	γ per 100 ml. red blood cells	*γ per cent*	*γ per cent*	*γ per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*
9-61	196	129	166		4.43	1.76	2.67
9-62	100	131	123		4.49	1.84	2.65
9-63	212	87	95		3.61	0.83	2.78
10-24	110	157	177	227	4.55	1.32	3.23
10-25	79	175	101	166	5.60	1.35	4.25
10-27	113	131	168	283	4.48	1.29	3.19
10-29	80	144	103	303	5.15	2.30	2.85
10-33	87	115	110	160	3.55	0.68	2.87
10-34	137	197	60	205	5.95	1.24	4.71
10-35	100	137	116	266	3.81	1.02	2.79
10-37	154	129	108	308	5.04	1.39	3.65
10-40	156	87	103	208	4.31	0.91	3.40
10-41	134	122	123	278	4.35	1.38	2.97
10-42*	224	113	19	269	3.24	0.93	2.31
10-43†	183	124	34	184	3.16	0.66	2.50
10-44	109	137	131	306	4.22	1.53	2.69
10-45	129	127	108	258	4.38	1.31	3.07
10-46	111	157	118	413	4.75	1.97	2.78
10-47	111	179	140	360	4.92	1.94	2.98
10-48	109	137	66	261	4.51	1.61	2.90
10-50	78	131	72	252	4.51	1.58	2.93
10-57	162	142	131	481	5.49	2.91	2.58
10-58	123	142	106	424	5.02	2.72	2.30
10-59	144	169	105	505	5.02	2.38	2.64
Mean.......	124	139	115	298	4.64	1.60	3.04

* Terminal hemorrhage; values not included in mean.

† Infection; values not included in mean.

tion in the iron-binding capacity. It is of interest that even though the iron-binding protein has been shown to be a globulin, the decrease in iron-binding capacity was correlated with the decrease in serum albumin rather than globulin. This may be explained by the fact that the iron-binding protein is a globulin of low molecular weight (90,000). It follows in great
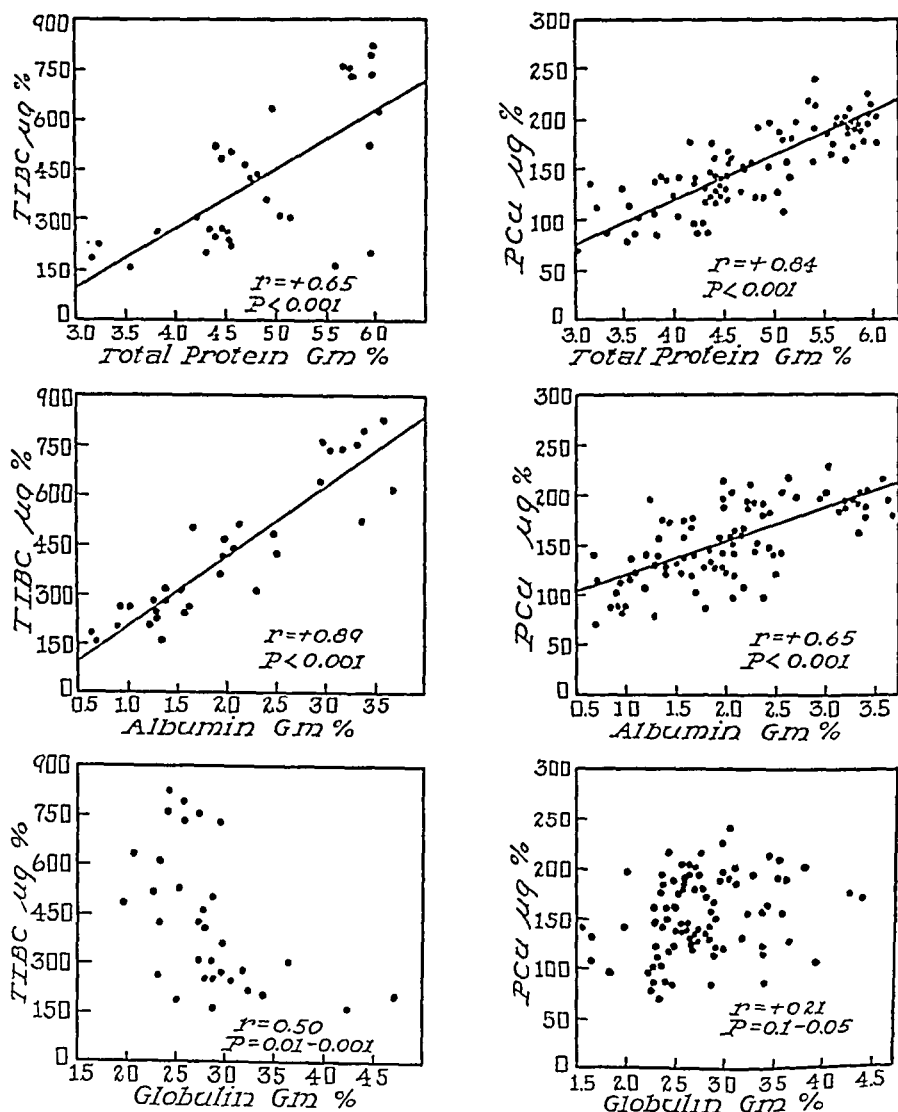
FIG. 1. The correlation between total serum protein, serum albumin, and serum globulin with the iron-binding capacity of the serum and the plasma copper. *r* refers to the correlation coefficient. *P* refers to the probability that a correlation as large as that indicated could occur by chance in a population in which no correlation exists. A value of *P* less than 0.05 is considered significant.

part the albumin fraction in the usual 23 per cent sodium sulfate fractionation procedure.

Hypocupremia was observed consistently in the hypoproteinemic ani-

mals.   The mean value was 139 ± 26.4 γ per cent as compared with a mean value of 206 ± 26.3 γ per cent in the control group.   The correlation between the plasma copper and total serum protein, albumin, and globulin is shown in Fig. 1.   The degree of hypocupremia appears to be correlated closely with the degree of hypoalbuminemia.

It is of interest that in the two pigs (Nos. 10-42, 10-43) with complicating disorders there was an increase in erythrocyte protoporphyrin and a decrease in plasma iron (Table II).   In one (Pig 10-42) there was a severe terminal gastric hemorrhage and in the other (Pig 10-43) a terminal infection accompanied by leucocytosis developed.   In human subjects both hemorrhage and infection are associated with a rise in erythrocyte protoporphyrin and a decrease in plasma iron (10).

TABLE III

Effects of Protein Therapy on Blood of Three Protein-Deficient Animals

Days of treatment 53.

Pig No.	Condition	Volume of packed red blood cells	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum	Total serum proteins	Serum albumin	Serum globulin
		ml. per 100 ml.	*γ per 100 ml. red blood cells*	*γ per cent*	*γ per cent*	*γ per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*
10-57	Deficient	33.8	123	127	123	480	4.48	2.49	1.99
	Treated	43.0	114	218	131	790	6.34	3.61	2.73
10-58	Deficient	35.0	123	124	99	425	4.42	2.06	2.36
	Treated	44.0	100	201	39	740	5.96	3.01	2.95
10-59	Deficient	30.0	115	161	105	500	4.55	1.67	2.88
	Treated	40.0	92	205	71	800	6.02	3.38	2.64

The effects of protein therapy in three protein-deficient animals are shown in Table III and the effects in a single animal are presented in detail in Fig. 2.   An increase in the casein content of the diet from 10 to 26 per cent produced a mild reticulocytosis, and was followed by an increase in the volume of packed red cells, plasma copper, iron-binding capacity, and serum proteins to normal.   There was no significant change in the erythrocyte protoporphyrin.   One animal (Pig 10-58, Fig. 2) developed persistent hypoferremia, probably because of the increased erythropoiesis, but in the other two the hypoferremia was only transient and was observed during the period of rapid blood regeneration.

Iron Deficiency—Morphologic and chemical findings in the pigs made iron-deficient are presented in Table IV.   The anemia was severe in degree, microcytic and hypochromic, and was accompanied by reticulocytosis. Significant alterations from the normal erythrocyte protoporphyrin and
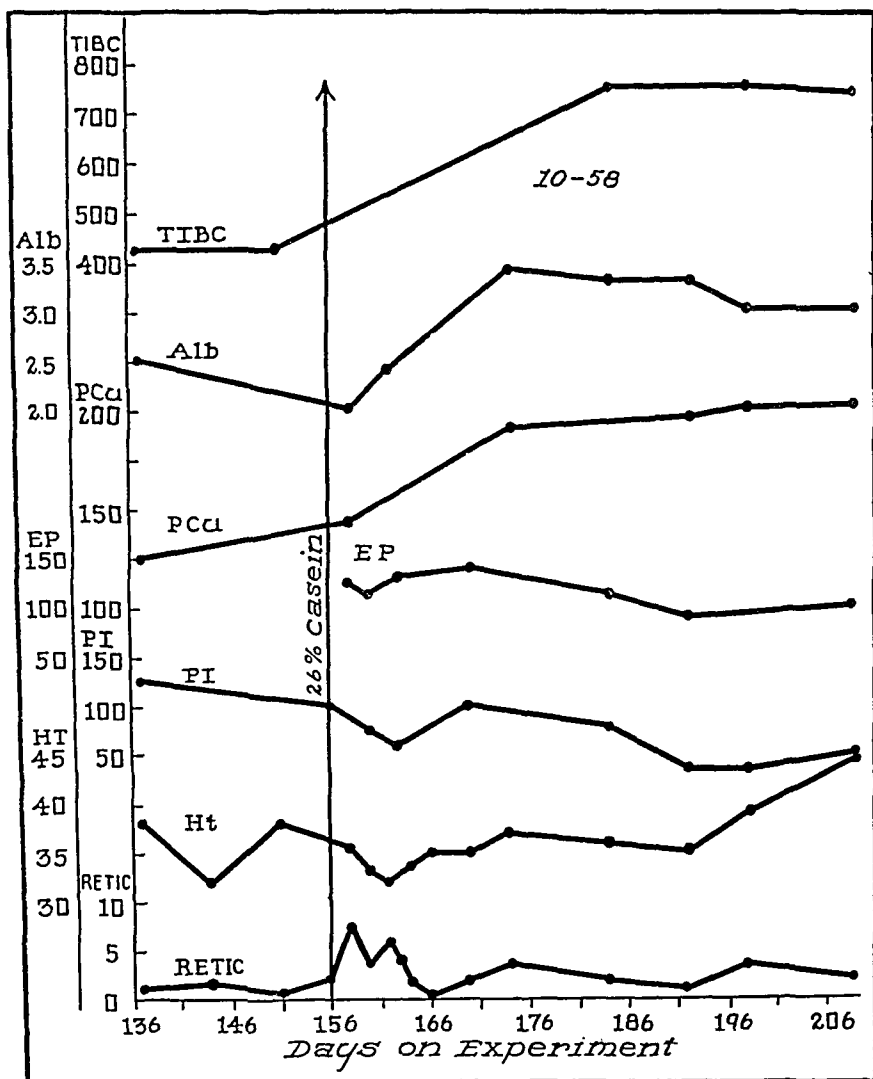
FIG. 2. The effect of protein therapy in a pig (No. 10-58) deficient in protein. The casein in the diet was increased from 10 to 26 per cent on the 156th day. *TIBC* represents total iron-binding capacity of the serum in γ per cent; *Alb* serum albumin in gm. per cent; *PCu* plasma copper in γ per cent; *EP* free erythrocyte protoporphyrin in γ per 100 ml. of red cells; *PI* plasma iron in γ per cent; *Ht* volume of packed red cells in ml. per 100 ml.; *Retic* reticulocytes in per cent.

plasma copper were not noted. The mean value for erythrocyte protoporphyrin was $127 \pm 31.4 \gamma$ per 100 ml. of red blood cells and for plasma copper $207 \pm 23.8 \gamma$ per cent. The anemia was, however, associated with

Table IV

Morphologic and Chemical Changes in Blood of Swine Fed Iron-Deficient Diets

Pig No.	Age at beginning of experiment	Days on experiment	Weight	Red blood cells	Hb	Volume of packed red blood cells	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Reticulocytes	Erythrocyte protoporphyrin	Plasma copper	Plasma iron
	days		kg.	million per mm.	gm. per cent	ml. per 100 ml.	cu. micra	micro-micro-grams	per cent	per cent	γ per 100 ml. red blood cells	γ per cent	γ per cent
9-13	35	175	75.5	7.23	7.0	27.0	38	9	26	5.8	95	216	32
9-14	35	160	59.2	6.27	6.9	25.5	41	11	27	6.3	140	257	38
9-24	21	152	25.6	6.13	3.7	18.5	30	6	20	4.4	90	201	17
9-25	21	163	53.6	6.29	5.2	21.5	34	8	24	6.2	125	199	24
9-26	21	194	54.5	4.26	3.7	15.0	35	9	25	6.6	105	206	40
9-27	21	194	65.4	5.36	5.1	19.5	36	10	26	1.2	86	189	41
9-46	21	63	25.9	4.30	3.9	15.5	36	9	25	4.2	174	198	26
9-47	21	64	27.0	5.94	5.5	22.0	37	9	25	2.8	116	193	24
9-48	21	78	26.3	4.05	3.0	12.5	31	7	24	5.4	177	187	29
9-49	21	78	23.4	3.78	2.4	10.0	26	6	24	4.6	178	177	29
9-50	21	78	30.6	4.60	4.0	16.5	36	9	24	7.2	116	177	32
9-51	21	78	48.8	5.43	5.4	20.0	37	10	27	5.8	141	221	26
10-16*	144	97	70.4	4.50	4.8	17.4	38	11	28	13.4	101	253	39
10-17*	144	97	75.2	6.20	5.2	21.0	34	8	25	18.4	113	209	30
10-18*	144	97	82.2	5.83	5.4	21.0	36	9	26	18.2	148	220	40
Mean..	47	118	49.5	5.34	4.7	18.8	35	9	25	7.4	127	207	31

* Hemorrhage superimposed on dietary deficiency of iron.

Table V

Effects of Acute and Chronic Hemorrhage on Blood of Three Pigs

Pig No.	Period	Volume of packed red blood cells	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum
		ml. per 100 ml.	γ per 100 ml. red blood cells	γ per cent	γ per cent	γ per cent
10-16	Control	42.2	105	195	152	
	Acute hemorrhage	17.2	180	264	16	
	Iron deficiency	17.4	101	253	39	340
	Treated (FeSO₄)	50.0	132	207	177	667
10-17	Control	40.0	120	209	105	
	Acute hemorrhage	20.0	239	293	27	
	Iron deficiency	21.0	113	209	30	392
	Treated (FeSO₄)	48.0	97	214	121	588
10-18	Control	37.6	124	197	200	
	Acute hemorrhage	20.8	239	286	29	
	Iron deficiency	21.0	148	220	40	890
	Treated (FeSO₄)	45.8	100	205	172	572

a severe hypoferremia, the mean plasma iron value being $31 \pm 7.2 \gamma$ per cent.

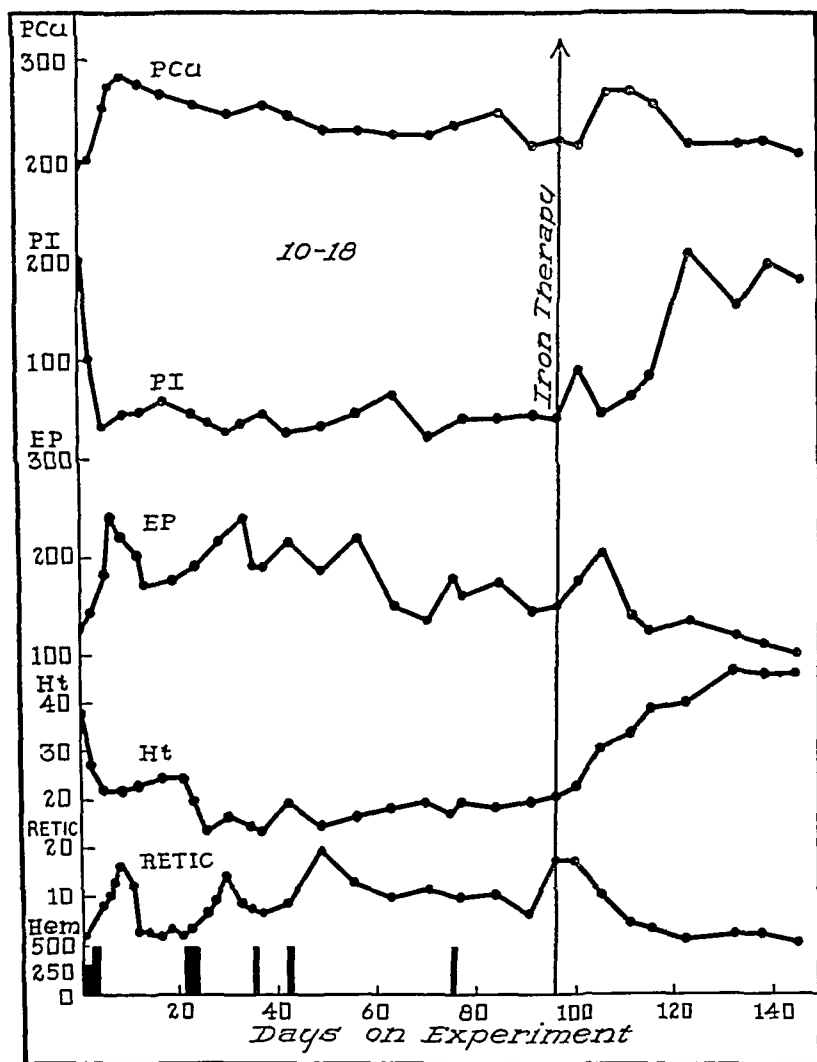FIG. 3. The effect of phlebotomy and a diet low in iron on the blood of a pig (No. 10-18). Under *Hem* the quantity of blood removed is expressed in ml. On the 96th day of the experiment the pig was treated with 2 gm. of ferrous sulfate daily. The legends are the same as in Fig. 2.

Three animals (Pigs 10-16, 10-17, 10-18, Table V) were fed a diet low in iron and 1500 ml. of blood were removed in 4 days. This resulted in all

three animals in severe normocytic normochromic anemia, reticulocytosis, severe hypoferremia, and a significant although not marked rise in erythrocyte protoporphyrin (219 ± 33.8 γ per 100 ml. of red cells) and plasma copper (281 ± 15.1 γ per cent). The effects of venesection are shown in detail for one animal (Pig 10-18) in Fig. 3. The low iron diet and phlebotomy were then continued until the animals developed severe microcytic hypochromic anemia. At this time the values for erythrocyte protoporphyrin and plasma copper were normal, although the hypoferremia persisted (Table V and Fig. 3). These animals were then treated with large doses of ferrous sulfate (2 gm. per pig per day) orally. In each animal there was a transient increase in erythrocyte protoporphyrin and plasma copper, followed by a decrease in reticulocytes and a return of all of the constituents studied to normal.

Determinations of the iron-binding capacity of the serum were made in only three iron-deficient pigs (Table V). In two of the animals the iron-binding capacity was reduced and in the third no change from the normal was noted. More data are needed before definite conclusions can be drawn, especially since the results are not consistent and are not in accord with those found in human subjects deficient in iron.[1]

DISCUSSION

Protein deficiency anemia was found to be accompanied by a severe hypoalbuminemia and a normal amount of free protoporphyrin in the erythrocytes. In view of the fact that protoporphyrin is believed to be synthesized from certain amino acid precursors (11) this finding is of some interest and would seem to indicate that porphyrin synthesis has priority over growth and the maintenance of normal serum proteins. This is in accord with the conclusion of Whipple and his group (12) that the body gives preference to hemoglobin production as compared with serum protein production. That severely hypoproteinemic animals can synthesize protoporphyrin readily is indicated by the fact that a substantial rise in erythrocyte protoporphyrin occurred in Pig 10-42 following a severe hemorrhage. Pig 10-43 responded in a similar fashion to an infection.

The slight reduction in plasma iron in the protein-deficient animals in comparison with normal control animals may be explained by the fact that the amount of iron carried in the serum is limited, at least under normal circumstances, by the amount of iron-binding protein in the blood (β_1-globulin, Fraction IV-7 of Cohn[1]). In the protein-deficient animals this protein appeared to be markedly reduced, since the mean iron-binding capacity was 298 ± 98.3 γ per cent as compared with 845 ± 90.6 γ per cent in ten control pigs. The mean per cent saturation of the protein with iron ((plasma iron)/(total iron-binding capacity of the serum)) for the deficient

animals was 38.6 and for the control animals 31.0. Thus it would seem that in both situations the iron-binding protein was maintained at about one-third saturation.

The moderate hypocupremia observed in the animals deficient in protein may indicate that the copper in the plasma is bound by a protein and that in the deficient animals this protein was markedly reduced. Since the degree of hypocupremia appeared to be correlated closely with the degree of hypoalbuminemia, this may indicate that the copper is bound to albumin or to globulin of low molecular weight. Cohn and his group have presented evidence that the β_1-globulin Fraction IV-7 may bind copper as well as iron reversibly although preference is given to iron.[2]

The fact that in protein deficiency there is no abnormality in erythrocyte protoporphyrin, only a slight reduction in plasma iron, and a significant reduction in plasma copper contrasts with the findings in the anemia of infection which is characterized by increased erythrocyte protoporphyrin, hypoferremia, and hypercupremia. It was suggested elsewhere (13) that the anemia of infection may be related to the disturbance in protein metabolism which accompanies trauma and various other types of tissue injury. The present observations indicate that the anemia of infection is not due simply to protein deficiency resulting from increased nitrogen excretion in the urine.

The normal erythrocyte protoporphyrin and plasma copper values in the iron-deficient pigs do not correspond with findings in patients with iron deficiency. Studies in this laboratory (10) have indicated that in human subjects microcytic hypochromic anemia due to a deficiency of iron is accompanied by a marked increase in erythrocyte protoporphyrin and plasma copper. The reason for this difference is not obvious. Two explanations can be suggested. Since the normal values for erythrocyte protoporphyrin and plasma copper in swine and man are quite different (1, 10) the failure of these to increase significantly in iron deficiency in swine may represent a species difference. Another possibility is that the high erythrocyte protoporphyrin values and hypercupremia which accompany microcytic hypochromic anemia in man are not due to iron deficiency *per se* but are due to some other cause. This is suggested by the fact that acute hemorrhage in both swine and man is accompanied by an increase in free erythrocyte protoporphyrin and plasma copper. Further work in various species under differing experimental conditions is needed before a definite conclusion can be drawn.

The results of the studies on erythrocyte protoporphyrin, plasma copper, and plasma iron in normal swine and in swine with anemia due to pyridoxine deficiency, protein deficiency, iron deficiency, and acute hemorrhage are

[2] Cohn, E. J., personal communication.

summarized in Table VI.   In general it may be concluded that pyridoxine deficiency is characterized by a microcytic normochromic anemia with a marked decrease in erythrocyte protoporphyrin, a slight reduction in plasma copper, and a marked increase in plasma iron.   Chronic protein deficiency is characterized by a normocytic normochromic anemia, normal erythrocyte protoporphyrin, a moderate hypocupremia, and a slight reduction in plasma iron.   Chronic iron deficiency is characterized by a microcytic hypochromic anemia, hypoferremia, and normal erythrocyte protoporphyrin and plasma copper.   Acute hemorrhage results in a normocytic normochromic anemia, an increase in erythrocyte protoporphyrin, and hypercupremia and hypoferremia.

TABLE VI

Summary of Data

Group	Type of anemia	Erythrocyte protoporphyrin	Plasma copper	Plasma iron	Total iron-binding capacity of serum
		γ *per 100 ml. red blood cells*	γ *per cent*	γ *per cent*	γ *per cent*
Normal	Normocytic, normochromic	118 ± 43.4	206 ± 26.3	169 ± 38.8	845 ± 90.6
Pyridoxine-deficient	Microcytic, normochromic	47 ± 13.6	160 ± 38.8	468 ± 166.6	
Protein-deficient	Normocytic, normochromic	124 ± 35.6	139 ± 26.4	115 ± 30.1	298 ± 98.3
Iron-deficient	Microcytic, hypochromic	127 ± 31.4	207 ± 23.8	31 ± 7.2	
Acute hemorrhage	Normocytic, normochromic	219 ± 33.8	281 ± 15.1	24 ± 7.0	

SUMMARY

1. Chronic protein deficiency in swine resulted in a mild normocytic normochromic anemia which was accompanied by normal erythrocyte protoporphyrin values (124 ± 35.6 γ per 100 ml. of red blood cells), slightly reduced plasma iron values (115 ± 30.1 γ per cent), a moderate hypocupremia (139 ± 26.4 γ per cent), and a marked reduction in the iron-binding capacity of the serum (298 ± 98.3 γ per cent).

2. Chronic iron deficiency in swine resulted in a severe microcytic hypochromic anemia which was accompanied by reticulocytosis, normal values for erythrocyte protoporphyrin (127 ± 31.4 γ per 100 ml. of red blood cells), normal plasma copper values (207 ± 23.8 γ per cent), and marked hypoferremia (31 ± 7.2 γ per cent).

3. Acute hemorrhage in swine resulted in a severe normocytic normo-

chromic anemia which was accompanied by reticulocytosis, an increase in erythrocyte protoporphyrin (219 ± 33.8 γ per 100 ml. of red cells), an elevation in plasma copper (281 ± 15.1 γ per cent), and marked hypoferremia (24 ± 7.0 γ per cent).

4. The results of morphologic and chemical studies of the blood of normal swine and of swine with anemia due to pyridoxine deficiency, protein deficiency, iron deficiency, and acute hemorrhage are summarized.

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ELECTROPHORETIC ANALYSES OF SERA OF NORMAL AND HYPOPROTEINEMIC SWINE*

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In the preceding paper (1) studies on free erythrocyte protoporphyrin, plasma copper, and plasma iron in protein-deficient and iron-deficient swine are reported. These experiments made animals available for electrophoretic studies which were severely hypoproteinemic as a result of a prolonged dietary restriction of protein. Studies on the sera of a number of these animals are presented in this paper.

Electrophoretic analyses of the sera of normal swine have been reported previously by Svensson (2), Moore (3), Deutsch and Goodloe (4), and Koenig and Hogness (5). No studies have been reported on the serum proteins of hypoproteinemic swine. However, Zeldis *et al.* (6) studied the electrophoretic patterns of the plasma of dogs following long and continued restriction of dietary protein. They found a marked decrease in albumin levels and essentially no change in the plasma globulin concentrations. The degree of depletion of "electrophoretic" albumin was considerably greater than that of "chemical" albumin. When large amounts of protein were fed to such dogs, complete restoration of the normal plasma albumin took place in several weeks. They concluded that "plasma globulins, in contrast to plasma albumin, enjoy prior demands on the total available pool of body protein materials under emergency conditions." They noted during depletion an increase in the total electrophoretic globulin, especially in the α-globulin areas, which was attributed to an increase in plasma lipides. Chow (7) in similar studies on dogs during chronic depletion by both plasmaphoresis and protein-free feeding observed similar changes. Studies in human subjects, hypoproteinemic as a result of malnutrition, have revealed a drop in the albumin content of the serum associated with a corresponding increase in α-globulins (7, 8).

Observations

The basal diet and vitamin supplements of the animals used in this study have been described in detail in the preceding publication (1). Three of

the control animals (Pigs 10-57, 10-58, 10-59) were maintained on a low protein (10 per cent casein) diet for 156 days. At this time the casein in the diet was increased to 26 per cent. The electrophoretic analyses were

TABLE I

Electrophoretic Analyses of Sera of Normal and Protein-Deficient Swine

The results are given as percentage of the total refractive increment. The num bers in parentheses refer to mobility $\times 10^{-5}$ sq. cm. per volt per second and are negative in sign.

Pig No.	Protein N	γ_2	γ_1	β_2	β_1	$\alpha_2 + \alpha_3$	α_1	Albumin
				Controls				
	mg. per ml.							
10-57	8.75	12	9	5	5	16	4	49
		(1.7)	(2.8)	(3.2)	(3.7)	(4.4)	(5.5)	(6.9)
10-58	8.27	9	13	5	2	19	4	48
		(1.9)	(2.8)	(3.4)	(3.8)	(4.7, 4.2)	(5.7)	(7.1)
10-59	11.33	13	12	5	3	20	3	44
		(1.6)	(2.5)	(3.1)	(3.4)	(4.1)	(5.1)	(6.1)
10-16	8.26		13*		16*	23	6	42
			(1.6)		(2.8)	(4.3)	(5.5)	(6.4)
10-17	13.37	21	7	4†	4	20	4	40
		(1.8)	(2.7)	(2.9)	(3.4)	(4.2)	(5.2)	(6.2)
				Protein-deficient				
10-40	4.89	28	6	3	6	39	10	8
		(1.9)	(2.7)	(3.0)	(3.3)	(4.0)	(5.5)	(6.5)
10-41	7.45		23*	6	7	38	8	18
			(2.0)	(3.1)	(3.5)	(4.6)	(5.7)	(6.8)
10-44	7.61	20	12		3	39	9	17
		(1.9)	(2.7)		(3.3)	(4.0)	(5.4)	(6.2)
10-45	5.59		23*	9	8	36	6	18
			(1.4)	(2.7)	(3.4)	(5.1, 4.4)	(5.8)	(6.3)
10-50	6.44	24	7	6†	1	36	2	24
		(1.6)	(2.7)	(3.3)	(3.6)	(5.0, 3.9)	(5.7)	(6.7)

* In these instances, the values represent total γ- or β-globulin, since the individual components could not be resolved accurately.

† Anomaly.

made on the 211th day of the experiment, 55 days after the protein had been increased to 26 per cent. The two other controls (Pigs 10-16, 10-17) were maintained on a diet containing 26 per cent casein from the beginning of the experiment. The electrophoretic analyses were made on the 240th

ay.   The hypoproteinemic animals were fed a diet containing 10 per cent asein for 160 to 261 days prior to the analyses.
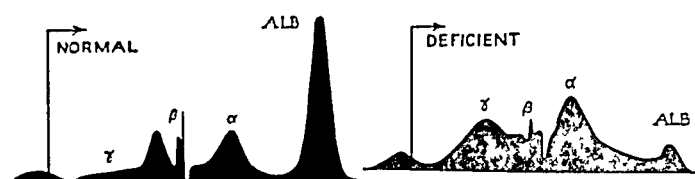

FIG. 1. Electrophoretic patterns of the sera of normal (Pig. 10-58) and protein-deficient (Pig. 10-40) swine. The deficient animal shows a marked diminution of lbumin and large relative increases of the globulin components. The photographs were taken of the descending boundaries at 250 minutes. The runs were performed t 1° in veronal buffer of pII 8.4 to 8.6 and at an ionic strength of 0.1.

TABLE II

Protein Composition of Sera of Normal and Protein-Deficient Swine

The values are calculated from the percentage for each component of the total efractive increment and the total protein content of the serum with the factor .7 × N. The concentrations are in gm. per 100 ml. of serum.

Pig No.	Total protein	γ_2	γ_1	β_2	β_1	$\alpha_2 + \alpha_1$	α_1	Albumin
				Controls				
10-57	5.85	0.70	0.53	0.29	0.29	0.94	0.23	2.86
10-58	5.55	0.50	0.72	0.28	0.11	1.05	0.22	2.66
10-59	7.60	0.99	0.91	0.38	0.23	1.52	0.23	3.34
10-16	5.54		0.72*		0.89*	1.27	0.33	2.33
10-17	8.95	1.88	0.63	0.36	0.36	1.79	0.36	3.58
Mean..	6.70	1.52		0.64		1.58		2.95
				Protein-deficient				
10-40	3.27	0.92	0.20	0.10	0.20	1.27	0.33	0.26
10-41	4.98		1.15*	0.30	0.35	1.89	0.40	0.90
10-44	5.10	1.02	0.61		0.15*	1.99	0.46	0.87
10-45	3.74		0.86*	0.34	0.30	1.35	0.22	0.67
10-50	4.51	1.08	0.32	0.27	0.05	1.62	0.09	1.08
Mean..	4.32	1.23		0.41		1.92		0.76

* In these instances, the values represent total β- or γ-globulin; the individual omponents could not be accurately resolved.

The electrophoretic analyses were made on sera at 1° in a Tiselius appa-atus equipped with the Longsworth schlieren scanning device. The sera were dialyzed for 48 hours against diethyl barbiturate (veronal) buffer at

pH 8.4 to 8.6 and at an ionic strength of 0.1.   The protein concentration in the cell was about 1.5 per cent.   Only descending patterns were measured.

In Table I our studies of the serum proteins of five normal and five hypoproteinemic swine are presented.   The values for the distribution and mobilities of the various components in the normal animals are in satisfactory agreement with those of previous workers (2–5).   The degree of hypoproteinemia is indicated in a more marked fashion by the amount of albumin than by the serum N values.   The average albumin concentration in the hypoproteinemic animals was 17 per cent as compared with 45 per cent for the controls.   The relative amount of α-globulin increased strikingly.   In the control animals the total globulins averaged 24 per cent and in the deficient animals 44 per cent.   A moderate increase in γ-globulins from 22 to 29 per cent was noted, but there was no change in the relative amount of β-globulin.   These changes are strikingly illustrated in Fig. 1 where the patterns obtained with the serum of a normal and of a hypoproteinemic animal are shown for comparison.

In Table II the absolute concentration in gm. per 100 ml. of serum for each of the serum constituents has been computed by multiplying mg. of protein nitrogen by 6.7 (9).   The data show a marked diminution in albumin from 2.95 gm. per cent in the controls to an average of 0.76 gm. per cent for the hypoproteinemic swine.   There was only a slight increase in the absolute amount of α-globulin and a moderate diminution in β- and γ-globulins.

Electrophoretic analyses of the plasma of these animals revealed no significant differences between the groups in the mobility or quantity of fibrinogen.   The mean content for each group was about 4 per cent with a range from 3 to 6 per cent.

DISCUSSION

Our results confirm those of Zeldis et al. (6) and Chow (7); namely, that a prolonged dietary restriction of protein results in a marked diminution in both the relative and absolute amount of albumin, while a relative increase occurs in globulin, especially α-globulin.   Previous electrophoretic analyses from this laboratory (10) on swine deficient primarily in tryptophan have revealed similar changes, although some alteration in the composition of the albumin was noted as indicated by a broad asymmetrical curve when acid-hydrolyzed casein was fed in place of crude casein.

It is of considerable interest that the degree of hypoproteinemia in these animals was much greater as determined by electrophoresis than by the chemical method (1).   It is also striking that, although there was a slight reduction in the β-globulin fraction as measured electrophoretically, the

metal-binding protein (β_1-globulin Fraction IV-7 (11)) as determined by the total iron-binding capacity was reduced about 65 per cent (1).

SUMMARY

Electrophoretic analyses have been performed on the sera of swine made hypoproteinemic by prolonged restriction of the dietary intake of protein and the results have been compared with the sera from control animals.

Chronic protein depletion in these animals results in a marked decrease in the absolute as well as relative amount of albumin and in a relative increase in the globulin fraction, especially in the α-globulins.

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STUDIES ON ADENOSINETRIPHOSPHATASE OF MUSCLE[*]

II. A NEW MAGNESIUM-ACTIVATED ADENOSINETRIPHOSPHATASE

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After Engelhardt and Lyubimowa (4) announced in 1939 that the ATPase[1] activity of muscle was contained in the myosin fraction, interest centered around the question of whether the enzyme was identical with the bulk of the myosin, was only a part of it, or was only adsorbed to it (see (1)). It was taken for granted by all investigators in this field that practically all of the ATPase activity was in this fraction. According to Needham (5), less than 10 per cent of the ATPase in muscle is water-soluble, the remaining activity being bound to the insoluble residue, the myosin fraction.

An evaluation of the experiments of Lohmann (6), who was the first to study the ATPase activity of muscle, showed that the water extract at neutral pH and in the absence of Ca can dephosphorylate ATP at a rate corresponding to more than 10 per cent of the activity of the whole muscle. However, this activity quickly disappears in about an hour at room temperature, and was therefore overlooked by all later authors.

We set about the task of isolating the enzyme fraction responsible for the activity of the fresh muscle extract. In spite of the instability of the enzyme this task was relatively successful. Although the purification has so far yielded preparations which, measured in absolute activity under optimal conditions, are not more concentrated than the best fractions obtained from the myosin enzyme (1), the purified enzyme can easily be distinguished from the latter by its different pH optimum, its specific activation by Mg and inhibition by Ca, and its different stability at various temperatures.

For Paper I of this series see (1); preliminary notes (2) and (3).

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[1] Abbreviations used, ATP = adenosine triphosphate; ADP = adenosine diphosphate; ATPase = adenosinetriphosphatase, splitting the first labile P group; pyro-P = 7 minute P minus direct P.

If both enzymes are compared under their optimal conditions of pH, activation, etc., characteristics in which they differ so greatly, the total yield measured by their activities is about equal. But under the conditions prevailing in the living muscle, neutral pH, high content of Mg, and absence of Ca in the interior of muscle, the new Mg ATPase is surely responsible for most of the dephosphorylation. In two respects it resembles the other ATPases, which so far have been obtained not only from muscle but from many other mammalian tissues: first, only the first labile P group of ATP is split off; secondly, it is a high molecular weight protein of globulin nature, forming complexes or adsorption compounds with the particulate matter, from which it cannot be easily separated.

For comparison with the results of other authors we followed the proposal of Bailey (7) and calculated the activity as Q_P; *i.e.*, microliters of H_3PO_4 split off per mg. of protein per hour at 38°. The protein was calculated from the nitrogen content with the factor 6.25. Incubations were for 5 minutes and phosphate is expressed as micrograms of P.

$$Q_P = \frac{\text{micrograms P} \times 22.4 \times 60}{\text{mg. N} \times 6.25 \times 31 \times 5}$$

Preparation of Mg ATPase—The animals (rats) were anesthetized with nembutal and killed by exsanguination. The carcasses were then chilled in ice prior to removal of the muscles of the hind·leg. The muscle was minced with scissors, suspended in 6 volumes of cold extracting solution (0.5 M KCl, 0.03 M NaHCO₃, 0.02 M Na₂CO₃, 0.001 M KCN), and ground in a Waring blendor for about 1 minute. The suspension was allowed to stand for about 20 minutes and then centrifuged at 4000 R.P.M. for 15 minutes. The residue was reextracted with the same volume of extracting solution as before. After 15 minutes extraction this suspension was centrifuged and the extracts combined. To remove the actomyosin the combined extracts were diluted with 6 volumes of cold 0.001 M KCN and the pH adjusted to 8.0 to 8.5. After standing about 30 minutes the suspension was centrifuged and the precipitate of actomyosin discarded. The enzyme was precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 35 per cent saturation (27 gm. of $(NH_4)_2SO_4$ per 100 ml.). The pH here and·in the subsequent reprecipitations was maintained at about 8.0 by addition of NH_4OH.

The $(NH_4)_2SO_4$ precipitate was centrifuged and then dissolved in 0.5 M KCl. The solution was centrifuged and the insoluble material discarded. The enzyme was then reprecipitated three times at 35 per cent saturation with $(NH_4)_2SO_4$. After each precipitation and resolution the preparation was clarified by centrifuging. After the last precipitation, the preparation was dissolved in a solution whose final composition was 0.06 M histidine,

0.4 M KCl, 0.001 M KCN. The pH should be about 7.5 at room temperature.

Starting with 25 gm. of muscle, at the dilution step at which the actomyosin was removed, the volume was somewhat greater than 2 liters. Since the handling of larger volumes was inconvenient, this was the usual weight of muscle employed. The final solution of the enzyme was then kept at 50 ml. and a 0.2 ml. aliquot of a 1:9 dilution of this solution gave 30 to 50 γ of P in the procedure for determining enzyme activity.

Though distilled water or dilute bicarbonate would extract some of the enzyme from muscle, the use of the stronger salt solution improved the yield considerably. However, since practically all of the muscle substance had been brought into suspension, it was necessary to remove the myosin or actomyosin. This was accomplished by dilution to an ionic strength of approximately 0.08. During the extraction and other manipulations the pH was maintained above 8. It has been found that up to the point of removal of the actomyosin the amount of enzyme obtained falls off sharply below pH 7.5. The amount of enzyme obtained in the $(NH_4)_2SO_4$ precipitation is also somewhat greater in alkaline solution.

When the procedure given here is followed, the quantity of enzyme obtained, when related to the weight of muscle, is sufficient to split off about 5 mg. of P per gm. of muscle in 5 minutes at 38°. By reextracting the actomyosin precipitate this may be increased by about 50 per cent, but the volumes of liquid to be dealt with do not make it worth while.

The enzyme may be further purified by high speed centrifugation. For this purpose the final solution of the enzyme was diluted with an equal volume of 0.5 M KCl and centrifuged at 10,000 $\times$ g for 20 to 30 minutes. The precipitate obtained usually contained about 15 to 20 per cent of the total activity but was generally discarded, for, though its purity was slightly greater than that of the original solution, it was considerably lower than that of the material obtained by subsequent centrifugation of the supernatant at higher speed.

The supernatant from this first centrifugation was then centrifuged at 18,000 $\times$ g for 20 minutes, and the supernatant was poured off and recentrifuged, while the precipitate was resuspended. This process was repeated until no more precipitate was obtained. Resuspension of the clear brown gel, which forms the pellets, is somewhat easier when repeated short periods of centrifugation are used rather than one period of an hour or more. The resuspended precipitates were all combined and completely dispersed by use of a hand homogenizer. As before, the solution was in 0.4 M KCl, 0.06 M histidine, and 0.001 M KCN. Finally, the preparation was centrifuged at 4500 R.P.M. for about 30 minutes and the precipitate rejected. This solution contained about 60 per cent of the total activity

of the original solution before high speed centrifugation. High speed centrifugation resulted in a 3-fold increase in purity on the average. The Q_P values were usually in the range of 6000 to 10,000. Some typical results are given in Table I. Some preparations have been subjected to a second centrifugation at 18,000 × g but the Q_P values were rarely improved by as much as 50 per cent.

The concentrated preparations of the enzyme are brown in color, usually somewhat pink, and are highly opalescent. Since the sedimentation at 18,000 × g would identify the material as the small particles or microsomes obtained from various tissues by Claude (8), it is not surprising that our preparations contain about 30 per cent lipide on the dry weight basis. They have a high phosphorus content, most of which is lipide P (85 to 90 per cent). There is a small variable amount of ribonucleic acid (0 to 10

TABLE I

Increase of Activity by High Speed Centrifugation

Initial (solution of $(NH_4)_2SO_4$ ppt.), Q_P	Centrifugation at 18,000 × g, Q_P	Increase × initial
2860	8,100	2.8
2790	7,350	2.6
3170	10,960	3.5
2410	7,360	3.1
2940	8,600	2.9
2270	6,700	3.0

er cent). A study of the distribution of phosphorus according to the method of Schmidt and Thannhauser (9) also indicates that about 5 to 10 per cent of the P is "phosphoprotein P;" the presence of desoxyribonucleic acid is questionable. Claude has suggested that the positive Schiff test given by his preparations, which we have observed also, is due to the presence of Feulgen's acetal phospholipides (10). The nucleic acid present in our preparations can be removed with ribonuclease without influencing the enzyme activity.[2] Attempts to separate the enzyme from the lipide material have been unsuccessful.

Though the preparations are quite unstable, the loss of activity is retarded by the presence of cyanide and some preparations have even been observed to increase in activity for a few days. In the case of two preparations this extended over a period of 8 days and the activity doubled in this time. However, these magnitudes were uncommon and the preparations usually did not retain their activity more than 7 or 8 days.

[2] We thank Dr. Kunitz for supplying a sample of crystalline ribonuclease.

In the clarification following each $(NH_4)_2SO_4$ precipitation, a black precipitate was obtained. On the basis of a pronounced test for Fe, this was presumed to be hemin. This fact, coupled with the appearance of the preparations, suggests that the cyanide effect is through formation of complexes with Fe and possibly other heavy metals.

The pH of maximum stability (about 7.5) is somewhat higher than the pH optimum of the enzyme. This is probably due to the effect of pH on CN^- concentration and loss of HCN from the solution.

Preparation of Myosin—The preparation of "crystalline myosin" given by Szent-Györgyi (11) was employed. It was found that myosin binds phenolphthalein rather strongly and, therefore, a pH meter was used rather than the indicator in that part of the procedure in which residual actin is removed.

Preparation of Actin—Bailey and Perry (12) found that the turbidity of actin prepared by the modified method of Straub (11) could be reduced by chloroform extraction of the acetone-dried muscle residue. We have made similar observations, using alcohol-ether extraction. Otherwise the preparation was identical with that of Straub.

Preparation of ATP—Our preparations, made from rabbit muscle, followed the procedure of Kerr (13) with modifications (1). For preparing a neutral stock solution, Ba and heavy metals were removed by Amberlite resin. A 30×0.8 cm. tube was filled with 15 gm. of Amberlite (washed with Na_2CO_3) and 250 mg. of Ba salt dissolved in 0.1 N HCl were washed through during about 30 minutes. The total volume was kept at 25 ml. and the final pH was 7.0 to 7.4. The solution could be kept in the ice box for weeks.

The amount of ADP in the ATP solution is usually determined by the ratio of 7 minute P to total P. A more accurate method consists of incubating an aliquot of the ATP solution with an excess of glucose and purified hexokinase from yeast. The transphosphorylation to glucose stops when the first labile P group has been transferred.

However, the hexokinase preparation must be free of phosphohexoisomerase, or a correction must be applied for the fructose-6-phosphate formed. This correction amounts to 5 per cent of the 7 minute P, if the equilibrium of isomerization is attained. A once crystallized preparation of hexokinase, kindly supplied by Dr. Kunitz, still contained some isomerase, which necessitated a correction of 1.5 per cent. By this method, our own preparations, freshly made, contained 90 to 96 per cent ATP, while the remaining 4 to 10 per cent of the labile P was ADP. A commercial preparation of the Na salt from Rohm and Haas, Philadelphia, contained as much as 28 per cent ADP; tested with two different preparations of the Mg ATPase, 35.5 per cent of the total 7 minute P was split off, an excellent agreement (see

Fig. 1).. It should be noted that with the usual procedure for developing the color of molybdenum blue, in the modified Fiske-Subbarow procedure for phosphorus, 2 per cent of the pyro-P is split. This inaccuracy can be avoided if the color is developed in the presence of ethyl alcohol (14) and then immediately read in an Evelyn colorimeter.

Measurement of Activity—Activity was determined in a system consisting of 1.0 ml. of buffer (0.1 M histidine or 0.05 M borate), 0.1 ml. of 0.15 M $MgCl_2$ or $CaCl_2$, 0.5 ml. of ATP (600 to 700 γ of 7 minute P per ml.), a suitable aliquot of the enzyme solution (usually 0.2 ml.), and H_2O to a total volume of 3.0 ml. Incubations were made at 38° for 5 minutes and the reaction stopped with 3.0 ml. of 5 per cent trichloroacetic acid. The solutions were then analyzed for inorganic phosphorus with the methods used in this laboratory.

Protein Determination—Nitrogen was determined by micro-Kjeldahl analysis according to the method of Ma and Zuazaga (15). Since the preparations contained ammonia as well as histidine, the protein was first precipitated with 10 per cent trichloroacetic acid, then twice resuspended in dilute trichloroacetic acid, and finally dissolved in N NaOH and transferred to the digestion flasks.

pH determinations were made with a Cambridge model L pH meter.

Factors Influencing Activity of Mg ATPase

Substrate Specificity—The Mg ATPase has no effect on β-glycerophosphate, hexose diphosphate, adenylic acid, and ADP. In the decomposition of ATP the reaction stops when one phosphate group has been removed. There appears to be no suppression of the activity by the accumulating ADP and the decomposition follows a curve for a first order reaction. Fig. 1 shows a plot of the data from one experiment. The curve was drawn on the basis of a total substrate concentration of 113 γ of P derived from the hexokinase analysis of the ATP solution. The rate constant was obtained from the data of two different enzyme preparations.

Influence of pH—The relationship between pH and activity of the Mg ATPase is shown in Fig. 2. The pH optimum is about 6.8 at 38°. On the acid side of the isoelectric point of histidine the pH of the buffer is reduced about 0.20 to 0.25 pH unit for a 10° rise in temperature. The effect of temperature on borate buffers is negligible.

The selection of a suitable buffer in the region of the pH optimum of the Mg ATPase presented some difficulties. The phosphate buffers ordinarily used around neutrality obviously could not be used here. The only other buffer in general use, acetate-veronal, has practically no buffer capacity in the region of pH 5.5 to 7.0. Though not generally used as a buffer, histi-

dine is quite good throughout the range of pH 5 to 11. The buffer solution was made up in 0.1 M concentration with the ionic strength adjusted to
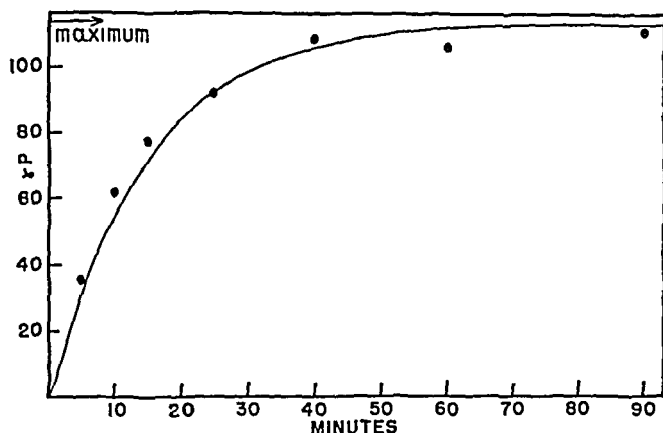
FIG. 1. ATP hydrolysis catalyzed with Mg-activated ATPase. 0.00736 mg. of protein nitrogen in 3 ml. Mg concentration = 0.005 M. First P group of ATP given by hexokinase analysis equivalent to 113 γ of P. For the curve, $k = 0.066$ min.$^{-1}$
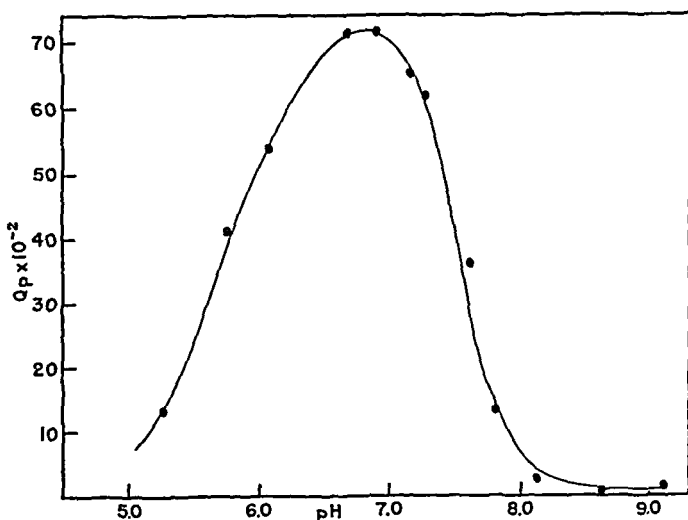
FIG. 2. Influence of pH on the activity of the Mg-activated ATPase. Incubation run in histidine buffer (0.03 M). Mg concentration = 0.005 M. Time, 5 minutes; temperature, 38°.

0.075 with KCl. There is no indication that the choice of buffer influenced the results as long as the pH was maintained.

With myosin, on the other hand, the only consistent results have been obtained in borate buffers. The mixtures of myosin and actin, however, seem somewhat less affected by the choice of buffer, and for the Mg activation of these mixtures the pH optimum in histidine is somewhat lower than in borate.

Activation and Inhibition—The new enzyme is activated by Mg and to some extent by Mn; Ca, Ba, and Co are without effect. Ca inhibits when both Ca and Mg are present in equal concentration. The relationship between activity and concentration of Mg, Mn, and Ca is represented in
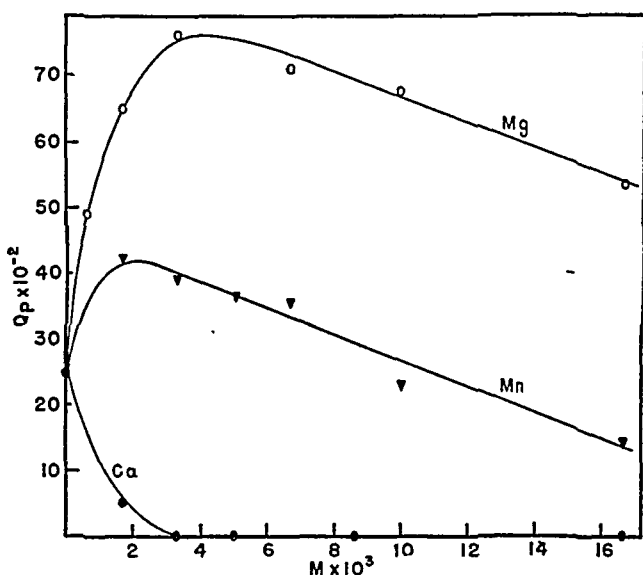
FIG. 3. Influence of Mg, Mn, and Ca on activity of Mg ATPase. Incubations in histidine buffer, pH 6.9, at 38° for 5 minutes. Total substrate equivalent to 113 γ of P (first group of ATP).

Fig. 3. There is always some activity in the absence of added Mg. Estimates of the dissociation constant for an Mg-enzyme complex have varied from 3.0×10^{-4} to 1.0×10^{-3}. The presence of other ions as well as the *ionic strength influences the activity to a minor degree. In the system for* measuring activity, the activity is optimal at an ionic strength of about 0.1. When the ionic strength is adjusted with K_2SO_4 rather than KCl, the activity is about 10 per cent greater with $SO_4^=$ than with Cl^-.

In addition to the inhibition by Ca, the enzyme is inhibited by fluoride and, judging by experiments with *p*-chloromercuribenzoate, sulfhydryl reagents will also inhibit. CN^- and N_3^- when added to the incubation mixture have a negligible effect on the activity.

Relationship between Activity, Enzyme Concentration, and Substrate Concentration—The activity is linear with enzyme concentration as long as the substrate concentration is non-limiting. The relationship between activity and substrate concentration appears to behave according to the concept of formation of an enzyme-substrate complex, though determinations of the Michaelis-Menten constant have not been highly reproducible, varying from 2.0 to 4.0 $\times$ 10^{-4}.
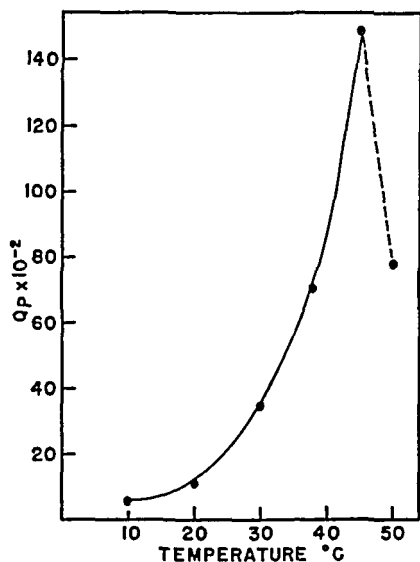
FIG. 4

FIG. 5

FIG. 4. Influence of temperature on the decomposition of ATP catalyzed by the Mg ATPase. Histidine buffer, pH 6.9 at 38° or pH 7.5 at 10°. Mg concentration = 0.005 M; total substrate equivalent to 113 γ of P (first group of ATP).

FIG. 5. Schematic representation of enzymatic behavior of myosin and myosin + actin. Solid line, myosin; dotted line, myosin + actin. The behavior is represented for borate buffers with the ordinate in $Q_P \times 10^{-2}$.
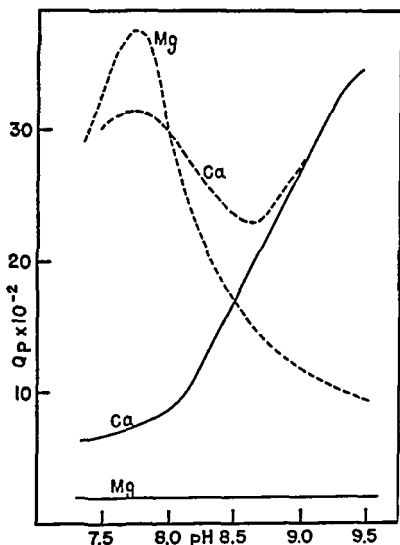
Influence of Temperature—The relationship between activity and temperature is reproduced in Fig. 4. The temperature coefficient is in the neighborhood of 2.6. At pH 7.15 the enzyme can withstand incubation at 38° for 1 hour without loss of activity. Myosin ATPase, on the other hand, loses its activity rapidly under these circumstances.

DISCUSSION

During the course of the investigation involving the Mg-activated ATPase the question of a possible relationship with the myosin system naturally arose. Szent-Györgyi (11) noted that myosin is Mg-activated

in the presence of actin.   We have given particular attention to this situation in order to determine whether or not it bears any relationship to our enzyme.   In addition, we have found changes in the behavior of myosin to Ca activation in the presence of actin.   The shape of the activity-pH curve changes, resulting in two nearly equal maxima.   The maximum around pH 9.5 does not change greatly but a new peak develops around pH 7.7 to 7.8.   Though variable, in some cases this has amounted to a 5-fold increase over myosin alone at this pH.   Because of the high viscosity of myosin-actin mixtures there are obvious difficulties attendant on the investigation of the enzymatic activity of these preparations.   However, from the results of a large number of experiments on this system a schematic representation of the behavior has been constructed and is reproduced in Fig. 5.   When histidine buffers are used rather than borate, the myosin-Ca curve is considerably depressed; the actomyosin-Ca relationship shows no alteration around pH 7.7 to 7.8 but the upper portion of the curve is depressed.   The actomyosin-Mg curve is displaced, the maximum occurring around pH 7.4.

It should also be pointed out that though the activity-pH relationship of actomyosin has been specifically studied in the presence of either Ca or Mg, several experiments run at pH 7.4 indicate that the presence of these ions has little effect on the activity.   This may be of considerable importance in the chemical mechanism of contraction, even though the physiological status of actin may not be clear.   Though all of our observations on the myosin-actin system agree in the qualitative sense, the quantitative aspects have been quite variable and appear to depend on the individual myosin and actin preparations.

We have not been able to observe any variations in the behavior of the Mg ATPase when either myosin or actin is added to it.   Nor have we been able to separate an Mg-activated component from myosin-actin mixtures. When the procedure used in isolating the Mg ATPase was applied to myosin-actin mixtures, the activity in the presence of Ca was completely recovered in the actomyosin, but a considerable portion of the activity in the presence of Mg was unaccounted for.

SUMMARY

An unstable Mg-activated ATPase has been isolated from muscle by extraction with dilute alkaline solution and repeated fractionation with 0.35 per cent saturated ammonium sulfate.   The enzyme is bound to particulate matter which is centrifuged at high speed.   It is free of myosin and actomyosin.

The enzyme splits off only one labile P group of ATP.   It has a pH optimum at 6.8 and is strongly inhibited by Ca.   The activity is around

Q_P 8000, and the total yield under optimal conditions is about equal to that of the myosin ATPase. So far no indications have been found that it is another form of the latter enzyme.

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PREPARATION AND CHARACTERIZATION OF DEXTRAN FROM LEUCONOSTOC MESENTEROIDES

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In conjunction with investigations on the α-1,6-glucosidic linkage in starch, it was desirable to conduct comparative studies on other substances containing this linkage. The dextran from *Leuconostoc mesenteroides*, a polysaccharide having predominantly α-1,6-glucosidic linkages, was selected to be used directly in these comparative studies and to provide a source of simpler substances containing this linkage of rare occurrence. Experiments were designed to provide a dependable source of dextran of high purity and of reproducible high viscosity. Improvements have been made over methods previously described for the preparation of this polysaccharide (1-4), and procedures have been established for the preparation of dextrans of low as well as of high viscosities.

Factors Influencing Dextran Production—Numerous factors, only a few of which have been studied (1, 4), appear to influence the properties and the amount of the dextran and of the by-products produced from sucrose by cultures of *Leuconostoc mesenteroides*. A factor of outstanding importance is the strain of the organism (5), which appears to determine whether the dextran is water-soluble or water-insoluble (3, 6). The structural basis for this difference in solubility is not known. Previously reported dextrans, most of which originated from different strains of *Leuconostoc mesenteroides*, have varied in other physical properties. Dextran has been obtained in yields of 18 (3) and 25 per cent (4), with specific rotations of +178° to +184° (2, 6, 7), of +195° (8), and of +198° (9). It has been reported to have high (2, 4, 6) and low (1, 9) contents of nitrogen, phosphorus, and ash. Some viscosity data for dextran are not on a comparable basis (7, 8) and other data present unexplained variations (5).

The optimum pH range for the enzymatic synthesis of dextran has been shown by Hehre to be 4.0 to 6.0 (7, 10). When preparing dextran from cultures of *Leuconostoc mesenteroides*, neutralization of the acidic by-products has been reported to increase the yield of dextran (1, 4, 11). The incubation time used previously for the production of dextran has

varied from 18 hours to 20 days (1–4). Methods reported for determining when dextran formation was complete have involved isolating and weighing the dextran from an aliquot of the medium (1) and measuring the viscosity of the culture medium (12).

In the present investigation, the data obtained are for *Leuconostoc mesenteroides* NRRL B-512 and its water-soluble dextran. Observations are reported here on the effect of pH and the composition of the culture medium, aeration, and incubation time on the yield and properties of this dextran. The manner of inoculation has been held constant.

Production of High Viscosity Dextran—The viscosity of dextran from *Leuconostoc mesenteroides* NRRL B-512 was found to be influenced greatly by cultural conditions. However, when these conditions were controlled, dextrans of high or of low viscosity were obtained as desired.

The procedure adopted for the preparation of dextran utilized an unaerated medium containing sucrose in 10 per cent concentration and buffered only with the mineral nutrients present. This was inoculated heavily with a rapidly growing culture of *Leuconostoc mesenteroides* NRRL B-512 and incubated at 25°. The course of dextran production was followed by measurement of the viscosity of the culture medium. The formation of dextran was paralleled by an increase in viscosity until, after about 24 hours incubation, the viscosity reached a maximum and dextran formation appeared to be completed. As is shown in Table I, these changes were accompanied by a decrease in pH from the initial value of about 7.0 to about 4.6 at the time of maximum viscosity.

Dextrans such as Dextrans A and D of Table II, which were isolated from the culture media at maximum viscosity, are called high viscosity dextrans. These dextrans after purification had characteristically high viscosities, highly positive optical rotations, and high purity.

The reproduction of results from preparations of high viscosity dextrans is demonstrated by Dextrans A and D (Table II) and by another typical high viscosity dextran which had a relative viscosity of 2.230 and was obtained in 26.8 per cent yield. Dextran B is not a typical high viscosity dextran (see Table I, foot-note).

In all cases, the products called dextrans were quantitatively precipitated as gummy masses by the addition of an equal volume of absolute ethanol to the culture medium. The technique used for isolating purified dextrans from aqueous solutions and for drying the product gave finely divided fluffy dextrans, which dispersed readily in water and underwent chemical reaction with ease when in the dry state.[1]

Production of "Autolyzed" Dextran—The viscosity of the culture medium containing dextran decreased when incubation was extended beyond the

[1] Jeanes, A., and Wilham, C. A., in preparation.

time of maximum viscosity. This decrease in viscosity, as is shown in Table I, was rapid at first and gradually became slower until a very low value was reached. During this incubation period, the pH of the culture medium decreased slowly from 4.6 to a steady value of 3.7.

Dextrans such as Dextrans C and E of Tables I and II, which were isolated from culture media of greatly reduced viscosity, are called "autolyzed" dextrans. In physical properties the "autolyzed" dextrans differ from the

TABLE I

Effect of Extended Incubation at 25° on pH and Viscosity of Unbuffered Culture Media

Culture Medium BC				Culture Medium DE			
Incu-bation time	pH	Absolute viscosity	Fraction isolated	Incu-bation time	pH	Absolute viscosity	Fraction isolated
hrs.		*centipoises*		*hrs.*		*centipoises*	
0	7.5	2		0	7.1	2	
23	4.9	60		23	4.6	203	Dextran D*
29	4.5	82		27.5	4.4	164	
32	4.45	75		32	4.3	131	
33	4.4	73	Dextran B*				
				47	4.0	74	
				72	3.9	43	
99	3.85	20		96	3.75	20	
				145	3.73	15	
191	3.7	11					
				273	3.7	10	Dextran E and other fractions
335	3.7	7					
384	3.7	6					
503	3.7	5	Dextran C and other fractions				

* These dextrans were isolated from one-half of their respective culture media, and the incubation of the other half was continued. When the isolation of Dextran B was actually begun, the viscosity of this part of the culture medium had decreased to 59 centipoises.

corresponding high viscosity dextran controls (Dextrans B and D) mainly in their lower viscosities. The viscosities of the "autolyzed" dextrans were inversely proportional to the duration of incubation; their yields were 2.1 to 2.5 per cent lower than those of their high viscosity controls.

Production of Dextran in Aerated and in Buffered Culture Media—Aeration of culture media was found to be unfavorable to dextran formation. Data on the pH and viscosity changes in an aerated, unbuffered culture medium are given in Table III, and the data on the product isolated therefrom, Dextran F, are given in Table II. As compared with the results from

unaerated culture media, aeration decreased the rate of formation, the yield, and the viscosity of the dextran, and did not prevent the culture medium from passing through a maximum viscosity. These results are not in conflict with the view of Hehre (10) that the action of the dextran-

TABLE II

Data on Dextrans from Various Culture Media

Dextran	Absolute viscosity of culture medium from which dextran was isolated	Properties of purified dextrans					
		Yield	N	P	Relative viscosity at 25°, 0.5 per cent in water*	$[\alpha]_D^{25}$ (in 1 N NaOH, C = 1)	Alkali No.†
	centipoises	*per cent*	*per cent*	*per cent*		*degrees*	
A	446	25.3‡	0.017	0.005	2.253	+203	0.0
B	73	24.7	0.033	0.008	2.003	201	0.0
C	5	22.2	0.022	0.011	1.414	199	0.6
D	203	23.7	0.032	0.007	2.133		0.1
E	10	21.6	0.010	0.004	1.565	200	0.3
F	14	<14.7			1.855	200	
G	847	24.0‡	0.000	<0.002	1.719	202	0.0

* The relative viscosities in 0.1 M calcium acetate of Dextrans A, C, and G were 2.235, 1.405, and 1.683, respectively. In 1.0 M calcium acetate, the value for Dextran A was 2.298.

† To serve as a basis for comparison, the following alkali numbers are quoted from (3): defatted corn-starch 11.0; corn amylose 20.2; corn amylopectin 5.9.

‡ The ash content of these representative dextrans was 0.02 per cent.

TABLE III

Effect of Aeration on pH and Viscosity of Unbuffered Culture Medium

Operation	Incubation time	pH of culture medium	Absolute viscosity of culture medium
	hrs.		*centipoises*
Aeration started*..............	0	7.5	2
" stopped	23.5	5.2	5
	32	4.7	12
	47.5	4.4	16
Dextran F isolated	55	4.3	14

* About 20 liters of air per hour were bubbled through the 6 liters of culture medium containing a small amount of lard oil to prevent foaming.

synthesizing enzyme does not appear to be "coupled with or dependent upon oxidative processes."

No advantage to dextran formation has been found by buffering the culture medium with calcium carbonate. Culture media buffered at pH

7.0 to 6.4 with calcium carbonate reached their maximum viscosity in 48 to 56 hours, and immediately the viscosity began to decrease rapidly. A dextran isolated from such a medium at its maximum viscosity is Dextran G of Table II. Comparison with the corresponding values for unbuffered preparations shows that buffering with calcium carbonate resulted in a marked increase in the viscosity of the culture medium, no increase in the yield, and a decrease in the viscosity but no decrease in the purity of the purified dextran.

Aerated media buffered with calcium carbonate did not pass through a maximum viscosity; the viscosity of such a medium increased slowly over a period of 30 days. Under these conditions the rate of dextran formation was decreased as compared with an unaerated, buffered medium.

Other Polysaccharide Fractions—In addition to dextran, culture media of *Leuconostoc mesenteroides* NRRL B-512 contained other polysaccharides in small amounts which were precipitated by adding ethanol to the media to make ethanol concentrations of 65 and 75 per cent. The amounts and properties of the fractions obtained varied with the conditions of production, but all fractions appeared to contain combined fructose. Some of the fractions were levans, and some became insoluble in water after one precipitation.

It has not been reported previously that *Leuconostoc mesenteroides* produces both dextran and levan. A few strains of *Streptococcus bovis* and of *Streptococcus salivarius* have been observed to produce both levans and dextrans (14).

EXPERIMENTAL

The medium used for growth of *Leuconostoc mesenteroides* NRRL B-512 and for the preparation of dextran was the same as that used by Hassid and Barker (2), except that 0.1 per cent of sodium chloride was added, as recommended by Tarr and Hibbert (1). The dipotassium hydrogen phosphate, in 5 per cent solution, was sterilized separately and added aseptically to the cool, sterile solution of the other components. Sterilization was effected by autoclaving at 15 pounds per sq. in. for 30 minutes.

Preparation of Inoculum—A culture of the organism was prepared by inoculating one standard loopful of rapidly growing stock culture into 125 cc. of sterile medium contained in a 300 cc. Erlenmeyer flask. This was shaken mechanically for 24 hours at 25°, and then transferred to 500 cc. of medium in a 3 liter Fernbach flask. After standing for 24 hours at 25°, this culture, totaling 625 cc., was transferred to 3 liters of the medium for the preparation of dextran.

The preferred incubation time of 24 hours for the 625 cc. inoculum was adopted on the basis of experimental observations. When 24 hour inocula

were used, culture media for dextran production reached high maximum
viscosities such as that of the typical Dextran A of Table II.   When 36
or 48 hour inocula were used, as for Dextrans D and B, respectively, pro-
gressively lower maximum viscosities of the culture media were obtained
(Table II).   It appears that the incubation time for the 625 cc. inocula in-
fluenced the viscosity of these unbuffered culture media.

Preparation of High Viscosity Dextran—12 liters of medium were steri-
lized in a 20 liter Pyrex bottle which was equipped with a siphon for the
aseptic withdrawal of test samples.   Incubation was at 25°.   The pH
values for this preparation culture were 7.1 after inoculation, 4.95 at 24
hours incubation time, and 4.75 at 26 hours.   At incubation times of 24 and
26 hours the absolute viscosity of the medium was 438 and 446 centipoises,
respectively.   The viscous material appeared to be homogeneously dispersed
in the cloudy culture medium and did not settle out.

Supercentrifugation of the culture medium was started at the end of
26 hours incubation.   The residue consisted largely of bacterial cells.
Absolute ethanol to make 35 per cent by volume was stirred into the cen-
trifugate, and the solution was again passed through the supercentrifuge
to remove the remaining small amount of bacterial cells.   The centrifugate
was stirred mechanically while the ethanol concentration was made up to
50 per cent by volume.   The dextran separated as a gummy mass from
which the supernatant was decanted.   The dextran was kneaded to remove
mother liquors, and was washed three times by kneading with 50 per cent
ethanol.   It was then dissolved in 11 liters of water and precipitated by
addition of an equal volume of ethanol.   The silvery looking mass was
again isolated, kneaded, and washed as before.   This cycle of reprecipita-
tion and washing was repeated twice more.

The dextran, redissolved in 2.5 liters of water, was precipitated by slowly
adding 100 cc. portions of the solution to 500 cc. of absolute ethanol, which
was agitated in a Waring blendor.   The precipitates were combined, col-
lected on a filter, washed twice by resuspension in 4 liters of absolute
ethanol, and filtered.   The product was dried *in vacuo* over anhydrous
calcium chloride at room temperature.   The weight of the product (dry
basis) was 361 gm., 25.3 per cent of the initial weight of sucrose, or 50.6
per cent of the glucose available from the sucrose.   Dextran A was shown
not to contain carbohydrates small enough to dialyze through Visking
cellulose membranes.   Other data on this product (Dextran A) are given
in Table II.

Preparation of "Autolyzed" Dextran—The changes in pH and viscosity
during the preparation of two "autolyzed" dextrans are given in Table I
and data on the purified products are given in Table II.   The time of in-
cubation of the 625 cc. inocula was 48 hours for Culture Medium BC and

36 hours for Culture Medium DE. 6 liter quantities of culture media were used. At 33 hours incubation, when Culture Medium BC was slightly past its maximum viscosity, one-half of it was removed aseptically and the high viscosity control, Dextran B, was isolated. The remaining half of the culture medium was allowed to stand at 25° for a total of 503 hours. The product insoluble in 50 per cent ethanol, "autolyzed" Dextran C, was then isolated in the usual way. In Culture Medium DE, the high viscosity control, Dextran D, was isolated from half of the culture medium at the time of maximum viscosity, and the "autolyzed" Dextran E was isolated from the remaining half of the medium after a total incubation of 273 hours.

The viscosities of Culture Media BC and DE, although of markedly different maximum values, decreased to 20 centipoises in 90 to 100 hours. After this time, the rate of change in viscosity was about the same in both media. At about 200 hours incubation, the pH of the culture media had reached a steady value of 3.7, and from then on changes in viscosity were very slow. This seems to be a practical time to isolate "autolyzed" dextrans.

Preparation of Dextran in Presence of Calcium Carbonate—Except as otherwise stated, all conditions and manipulations were the same as have been described for unbuffered culture media.

The 625 cc. inoculum contained 2 per cent of calcium carbonate and was incubated for 48 hours at 25° with occasional shaking. When this was transferred to the medium for preparation of dextran, 2 per cent sterile calcium carbonate was also added and kept suspended by occasional swirling during incubation of the culture medium. After inoculation, this culture medium had a pH of 7.0 to 7.1, and a viscosity of 2 centipoises.

When the culture medium was not aerated, the pH values were 6.5 at 22 hours and 6.4 at 47 hours; the corresponding viscosities were 4 and 879 centipoises, respectively. At 50 hours, isolation of the dextran was started. The culture medium was diluted with about one-third its volume of water, and supercentrifuged. The pH of the centrifugate was adjusted to 4.4 with acetic acid, and absolute ethanol was added to give an ethanol concentration of 35 per cent by volume. The mixture was then passed twice through the supercentrifuge and the dextran, isolated in the usual way, gave a 24 per cent yield. Other data for this Dextran G are given in Table II. The low nitrogen and phosphorus contents indicate that the procedure for purification of this dextran was more efficient in removing bacterial cells than that described for high viscosity dextran.

When the culture medium was aerated, the viscosities at 42, 50, 66, and 72 hours incubation time were 29, 122, 580, and 768 centipoises, respectively. During this time the pH was near 5.8. After 90 hours, when the

viscosity was 786 centipoises and the pH was 6.0, the dextran was isolated. The yield was 22 per cent, and the relative viscosity of the dextran was 1.666.

Effect of Sterilization on Decrease in Viscosity—An experiment was conducted to determine whether a decrease in viscosity would occur in a dextran preparation medium in which bacterial and enzyme action had been stopped by autoclaving. The usual unbuffered medium was inoculated with a 625 cc. inoculum which had been incubated 44 hours. After 24 hours incubation, the pH was 4.65 and the viscosity was 171 centipoises. The medium was autoclaved at 15 pounds per sq. in. for 30 minutes, and cooled quickly. The pH was still 4.65 and the viscosity was 138 centipoises. The pH was adjusted to 4.2 with sterile butyric acid solution, and the mixture was kept at 25° for a time which is expressed as a continuation of the incubation period. The pH remained constant, and the viscosity at 50, 121, and 174 hours was 135, 129, and 125 centipoises, respectively. Although a slow change in viscosity occurred under these conditions, the viscosity of this solution at 174 hours incubation was roughly 10 times the viscosities observed at comparable times for media in which normal autolysis had occurred (see Table I).

Effect of Variation of Medium—When the usual unbuffered medium was supplemented with 0.5 mg. of manganous sulfate monohydrate per liter (15), the incubation times for the two inocula and for the preparation culture medium were 16, 9, and 6 hours, respectively. The purified dextran, obtained in 24 per cent yield, had a specific rotation of +200.1° and a relative viscosity of 2.255. By using an unaerated, calcium carbonate-buffered medium to which 1 mg. of manganous sulfate monohydrate per liter had been added, the incubation time was 13 hours for both of the inocula as well as for the preparation culture medium. The purified dextran, obtained in 27 per cent yield, had a specific rotation of +200.8° and a relative viscosity of 1.673. Extension of the incubation time resulted in inactivation of the bacteria in the unbuffered inocula and in a decrease in viscosity of both the buffered and the unbuffered culture media.

Substitution of the mineral constituents of Dunn *et al.* (16) (with only 0.1 the concentration of ferrous and manganous sulfates recommended) for the mineral constituents of our medium resulted in no significant increase in dextran production. Inclusion in the medium of corn steep liquor, autolyzed yeast, or Bacto-tryptone appeared to be of no advantage.

Other Polysaccharide Fractions—From the 50 per cent ethanolic dextran mother liquors, after supercentrifugation to remove a small amount of dextran, fractions insoluble in 65 and 75 per cent ethanol were successively precipitated. The fractions were purified and isolated as white powders in a manner analogous to that described for dextran.

The fractions from unbuffered "autolyzed" media (see Table I) were homogeneously water-soluble, and their yields totaled 5 per cent of the initial weight of sucrose in the medium; dialysis reduced their nitrogen and phosphorus contents from about 0.04 and 0.20 per cent, respectively, to 0.02 per cent, but did not effect any significant change in other properties. These fractions had $[\alpha]_D^{25} = +55°$ to $+133°$ (in 1 N sodium hydroxide, $C = 1$), relative viscosities of 1.060 to 1.134 (0.5 per cent concentration in water, 25°), alkali numbers of 4 to 13, and contained combined fructose. Exposure in air having 100 per cent relative humidity at 25° converted them from a state which produced amorphous x-ray patterns to states from which x-ray diffraction line patterns characteristic of dextran were obtained (17).

The fractions from unbuffered, high viscosity culture media totaled about 4 per cent in yield. One fraction, isolated in 2.6 per cent yield, had $[\alpha]_D^{25} = -23°$, an alkali number of 1, and produced only amorphous x-ray patterns (17). Another fraction, which became water-insoluble during isolation, gave a dextran x-ray line pattern without further treatment.

Fractions totaling 3.4 per cent in yield were obtained from a calcium carbonate-buffered culture medium. A fraction, obtained in 2.8 per cent yield, gave $[\alpha]_D^{25} = -54°$ and an alkali number of 0.

Tests for ketose, which is assumed to be fructose, in these fractions were made by allowing some of the dry carbohydrate to stand in 85 per cent phosphoric acid at room temperature (18). Under these conditions, fructose, sucrose, inulin, and calcium 5-ketogluconate developed dark brown to black colors within 24 hours, but neither glucose nor dextran produced any color. Some fractions produced dark brown to black colors, and others produced light yellow to tan colors when tested in this way. The intensity of color appears to indicate the relative amount of combined fructose present. Further evidence of the presence of much fructose in fractions which produced dark colors was provided by their negative or low positive optical rotations.

Preparation of Water-Insoluble Dextran—For comparison with the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512, the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 was prepared. Our usual unbuffered sucrose medium was used. Because of the slowness of growth of the organism, 48 hours incubation was required. The culture medium became viscous with insoluble gelatinous particles. Microscopic examination revealed a heavy growth of non-capsulated bacteria, and discrete particles of gelatinous material.

The culture medium was diluted with an equal volume of water and centrifuged at 3300 R.P.M. The residue, which was insoluble in boiling water, was dissolved in 0.75 N potassium hydroxide. This solution was neutralized with acetic acid and supercentrifuged twice to remove bacteria.

Addition of an equal volume of ethanol precipitated the polysaccharide and rendered it insoluble in water. The precipitate was washed with 50 per cent ethanol and the polysaccharide, called Fraction J, was isolated in the usual manner as a coarsely fluffy product.

The supernatant from the diluted culture medium was supercentrifuged and an equal volume of ethanol was added. The precipitate, which swelled greatly in water but did not dissolve, was washed with 50 per cent ethanol and the polysaccharide, called Fraction K, was isolated from absolute ethanol.

The yields of Fractions J and K were 9 and 7 per cent, respectively, of the initial weight of sucrose. Fraction J gave $[\alpha]_D^{25} = +208°$ (in 1 N sodium hydroxide, $C = 1$). Both fractions gave negative tests for fructose in 85 per cent phosphoric acid. The percentage of nitrogen in Fraction J was 0.008, and in Fraction K 0.020.

Analytical Methods—Because anhydrous dextran is very hygroscopic, samples were equilibrated with moisture in a constant humidity room (50 per cent relative humidity at 25°) where all weighings were made. Under these conditions the moisture content of the dextrans was 12 to 13 per cent. The moisture content was determined on separate samples, and all results were calculated on a dry basis.

Dextran, which was always dried *in vacuo* over anhydrous calcium chloride at 25° before being equilibrated in 50 per cent relative humidity, was shown by ethoxyl determination to contain no ethanol.

Viscosity measurements were made with standardized Ostwald-Cannon-Fenske viscosimeter tubes, at 25° ± 0.03°. For measurements on purified dextrans, 0.5 per cent aqueous solutions were used after filtration through fritted glass funnels to remove traces of lint. For measurements on culture media, care was taken to obtain representative samples, and samples from calcium carbonate-buffered media were filtered through fritted glass funnels before use.

Optical rotations were read with the light from a sodium vapor lamp. Solutions in 1 N sodium hydroxide were used to avoid the opalescence which high viscosity dextrans gave in water solutions. However, specific rotations in water were only a few degrees lower than those in sodium hydroxide.

Measurements of pH were made with a glass electrode. Alkali number measurements were made by the method of Schoch and Jensen (19). The values are reproducible to ±0.3. Nitrogen analyses were made by the micro-Kjeldahl procedure, and phosphorus analyses by a modification of the method of Truog and Meyer (20).

DISCUSSION

Viscosity and Solubility of Dextran—Our results show that the maximum viscosity of culture media of *Leuconostoc mesenteroides* NRRL B-512 does

not give an accurate indication of the yield or viscosity of the pure dextran after its isolation.   Dextrans with relative viscosities of 2.003 to 2.253 have been isolated in yields of 24 to 25 per cent from unbuffered culture media having viscosities of 73, 202, and 446 centipoises.   These differences in viscosity of the culture media appear to be related to the age of the inoculum used.   The purified dextrans showed no significant difference in any other of the observed properties.   From a calcium carbonate-buffered culture medium with a viscosity of 847 centipoises, dextran having a relative viscosity of 1.719 was isolated in 24 per cent yield.   The very high viscosities of culture media buffered with calcium carbonate do not appear to be due to the effect of calcium ions on dextran alone, as is indicated by viscosity measurements on purified dextrans in solutions of calcium acetate (see Table II, foot-notes).

The viscosities of the purified water-soluble dextrans do not correlate with their nitrogen contents.   The slight differences in nitrogen content are believed to reflect variation in the efficiency of separation of bacteria from the dextran.   Likewise, the solubilities of our dextrans are not related to their nitrogen contents, for the purified water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 has a nitrogen content as low as the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512. It is inconceivable that the methods employed for the isolation and purification could have contributed to the solubility of the water-soluble dextran.   The solubility and viscosity of these dextrans apparently are inherent properties of the polysaccharides rather than related to combinations of the dextran with protein, as postulated by Stacey (21–23).

Causes and Effects of Decrease in Viscosity of Culture Media—The decrease which occurred in the viscosity of culture media after the formation of dextran was complete appears to be caused mainly by autolysis.   The change was almost, but not completely, stopped by heat sterilization of the culture media.   This viscosity change was not dependent on the pH or on aeration in unbuffered media.   Aeration of buffered media prevents it.

It is not yet known whether "autolysis" results in some selective structural change in dextran, or merely produces a random decrease in molecular size.

Stability of Dextran to Alkali—The alkali numbers in Table II show that Dextrans A, B, and D are not attacked when heated with 0.1 N sodium hydroxide solution, and that Dextrans C and E are only slightly attacked. From other reactions previously reported (2, 3, 9), evidence is available which also indicates the lack of reducing power in dextran.   According to the interpretation of alkali numbers (19, 24), our preparations of dextran have very few, if any, reducing groups.

From this viewpoint it is interesting to consider possible modes of termination of dextran molecules which would result in the stability of dex-

tran to alkali.   An unterminated cyclic structure, such as is found in the Schardinger dextrins, would be stable to alkali (13), but a cyclic structure for the molecule as a whole would not be in accordance with the pronounced filiform characteristics of water-soluble dextran (8, 17).   A chain structure terminated by a unit of glucose-1-phosphate (25), of fructose combined as in sucrose (19), of a hexahydric alcohol such as mannitol, or of gluconic acid (19, 24) would be stable to alkali.   Glucose-1-phosphate has been reported to be formed from sucrose by *Leuconostoc mesenteroides* (26), but there is no evidence that it takes part in the synthesis of dextran (27, 28).   The possibility that sucrose may be converted directly into dextran without formation of a simpler intermediate (28) could result in the dextran molecule having a terminal fructose unit.   No additional evidence can be cited for terminal glucose units in an oxidized or reduced state.

SUMMARY

1. A method has been established for the preparation of water-soluble dextran products of uniformly high viscosities and of high purity from cultures of *Leuconostoc mesenteroides* NRRL B-512 on an unbuffered, unaerated, sucrose medium.   This method was dependent on the correlations that the development of maximum viscosity in the culture medium coincided with the end of dextran formation and that thereafter the viscosity of the culture medium and of the dextran decreased.

2. Purified dextrans which were isolated from culture media at their maximum viscosity were characterized by high viscosities; purified dextrans isolated from culture media after their maximum viscosity had been passed had lower viscosities.

3. Accompanying the changes in viscosity of the culture medium, the pH decreased from an initial value of about 7.0, through about 4.6 at the time of maximum viscosity, to a steady value of 3.7 when the viscosity had become very low.

4. Modification of the selected cultural conditions by aeration of the medium or by buffering with calcium carbonate, either singly or in combination, gave no increase in the yield or viscosity of dextran.

5. The solubilities of the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512 and of the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 appear to be inherent properties of the polysaccharides and are not related to their nitrogen contents.

6. In addition to dextran, levan was produced in relatively small amounts in cultures of the strain NRRL B-512.

The use of trade names in this paper does not necessarily constitute endorsement of these products or of the manufacturers thereof.

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MOLECULAR ASSOCIATION IN DEXTRAN AND IN BRANCHED AMYLACEOUS CARBOHYDRATES

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PLATE 3

A property of polysaccharides which is of major importance both practically and fundamentally is orderly association between portions of the same or of adjacent molecules. This property depends upon both the physical and chemical constitution of the polysaccharide and data on it provide a means for correlating physical properties with chemical structure. Little specific information is available on molecular association in branched polysaccharides. The industrial importance of starch and the biological significance of glycogen and certain bacterial polysaccharides make such information desirable.

This paper presents information on molecular association in the bacterial polysaccharide, dextran from *Leuconostoc mesenteroides*, and in branched amylaceous substances. Orderly molecular association has been detected by x-ray analysis, and the tendency towards orderly association has been related to the physical and chemical constitution of these polysaccharides. Data are given on typical x-ray diffraction powder patterns of dextran, and on the relation of molecular association to the hydration and ease of dissolution of branched polysaccharides.

Chemical and Physical Nature of Dextran—Dextran is known to be a polymer of α-D-glucopyranose (1). Methylation studies on a water-soluble dextran (2) have shown that the molecule has a branched structure in which the predominant glucosidic linkage is 1,6- while 1,4-linkages occur at the points of branching. A point of branching for every 5 glucose units was indicated by these data. Less quantitatively exact methylation studies on other dextran preparations have indicated fewer side chains (1, 3). In amylaceous substances the predominant glucosidic linkage is α-1,4- while 1,6-linkages occur at the points of branching (4). The presence of 1,6-linkages in starch makes further knowledge of other polysaccharides containing this linkage desirable for comparative purposes.

Dextran has been shown to be filiform in nature by electron microscopy (5), by ultracentrifugation (6), and by birefringence of flow (6). Grönwall and Ingelman (6) reported that the molecular weight of the dextran studied by them was of the order of "many millions," but accurate evaluation was prevented by the inhomogeneity of size of the molecules.

Dextran molecules appear to differ in size (7) and probably in some details of chemical structure when prepared under different conditions and by different strains of *Leuconostoc mesenteroides*. Our observations on the water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512 which was used in this study indicate that, with the possible exception of the degree of branching, this dextran appears to conform to the structural characteristics indicated above. It cannot be assumed that the methylation data available (2) apply exactly to our dextran.

Molecular Order and Association—Samples of polysaccharides which produce x-ray diffraction powder patterns containing a set of diffraction lines are called "crystalline." A crystalline polysaccharide is ordinarily a mixture of regions which range in degree of molecular order between the theoretical extremes of complete crystallinity and complete randomness. A single polysaccharide molecule may extend through several different regions (8). In the crystalline regions segments of primary valence chains are in ordered spatial arrangement where energy relations favor systematic lateral association between chains through hydrogen bonds. In amorphous regions chain segments are in disorderly arrangement. X-ray patterns from ordinary crystalline polysaccharides are composites of the diffraction lines which originate in the crystalline regions and of the halo of diffuse reflections from the amorphous regions. Amorphous polysaccharides, which produce only diffuse x-ray reflections, are believed to vary in the extent and in the degree of randomness of molecular association.

EXPERIMENTAL

Materials and Methods—Unless otherwise stated, the dextran used has been the water-soluble product from *Leuconostoc mesenteroides* NRRL B-512 and was prepared and characterized as described in a previous study (7).

Humidification in an atmosphere having 100 per cent relative humidity at 25° has been used to convert amorphous samples of glutinous cornstarch, amylose-free corn amylopectin, and certain fractions from acid-hydrolyzed dextran to the crystalline state. Dextrans and acid-hydrolyzed dextrans have produced line patterns after treatment under special conditions with specific optimum concentrations of aqueous ethanol. Crystallization has also been found to develop in dextran when its aqueous pastes were dried at elevated temperatures. These general methods, or the fundamental principles underlying them, have been used previously in studies on starch (9, 10) or other polysaccharides (11).

X-ray patterns were made with unfiltered copper radiation transmitted through the finely pulverized samples which were packed in holders 1 mm. thick. Before the pattern was taken, all samples which had been stored several days in an atmosphere having 100 per cent relative humidity at 25° were permitted to dry in the air from 1 to several hours to reach a satisfactory condition for mounting. The exact state of hydration of samples when x-rayed has not been defined.

Amorphous Dextran—Dextran is normally isolated as a fluffy white solid (7) which gives an amorphous x-ray pattern, and in which the randomness of molecular association is very high. Unless otherwise stated, amorphous dextran has been the starting material used in all the experimental work described here.

Amorphous dextran takes up 12 to 13 per cent moisture when equilibrated in an atmosphere having 50 per cent relative humidity at 25°. When a small amount of amorphous dextran was exposed in an atmosphere having 100 per cent relative humidity at 25°, it changed to a clear, thick syrup within 2 to 3 hours. After 3 days under these conditions, x-ray examination showed the dextran still to be amorphous. These observations have been made on dextrans with relative viscosity[1] of 2.003, 1.816, and 1.414.

X-ray Data and Patterns—Three general types of x-ray diffraction powder patterns, one of which has two modifications, have been obtained from dextran and acid-hydrolyzed dextran. The x-ray data for these typical patterns are given in Table I and illustrations are given in Fig. 1.

Preparation and Properties of Samples

Dextran Treated with Aqueous Ethanol—0.5 gm. samples of dextran (relative viscosity[1] 1.816) were dissolved in 15 cc. of distilled water in small glass-stoppered flasks, and absolute ethanol was added to make ethanol concentrations of 40, 50, 60, 70, and 80 per cent by volume, respectively. The dextran precipitated and was allowed to stand under the aqueous ethanol for 7 days at 25°. The precipitates were then removed and dried *in vacuo* over anhydrous calcium chloride at 25°. When dry, the sample from 60 per cent ethanol pulverized with great ease and the sample from 70 per cent ethanol pulverized easily, but all the other samples could be broken only into lumps. The samples from 40 and 50 per cent ethanol produced amorphous x-ray patterns, those from 60 and 70 per cent ethanol produced line patterns of type L-1, and the sample from 80 per cent ethanol gave a pattern with traces of lines on a diffuse background.

Humidification for 3 days in an atmosphere saturated with water vapor at 25° produced no apparent change in the dry, pulverized samples from 60 and 70 per cent ethanol, but resulted in increased sharpness of the type

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[1] Viscosities were measured on 0.5 per cent solutions in water at 25° (7).

L-1 patterns produced. When humidified, the sample from 40 per cent ethanol dissolved readily to a clear syrup, which, after drying, produced an amorphous pattern. Similarly, the samples from 50 and 80 per cent ethanol formed cloudy syrups, which, after drying, produced amorphous x-ray patterns showing traces of lines.

Extension of the time of treatment in aqueous ethanol to 35 days resulted in an amorphous product from 40 per cent ethanol and a crystalline product from 50 per cent ethanol.

TABLE I

Interplanar Spacings and Estimated Intensities of Typical Dextran Patterns

Type L-1		Type L-2		Type L-3		Type L-3'	
A		A		A		A	
13.0	Weak					13.0	Very weak
6.50	"			6.93	Medium	7.80	" "
5.76	Medium	5.65	Very weak	6.13	"	6.02	Medium
5.05	"	4.95	Medium	5.01	"	5.05	Weak
4.69	"	4.52	"	4.52	Very strong	4.55	Strong
4.32	Medium strong	4.00	Very, very weak			4.25	Very, very weak
.90	Very weak			3.89	Strong	3.90	Medium
.56	" "	3.57	Very weak	3.49	Medium	3.47	"
.33	Medium	3.24	Weak				
.04	"	3.06	Very, very weak	3.11	Very weak		
.79	Very, very weak			3.00	Weak	3.04	Medium
.65	" "	2.55	Very, very weak	2.84	Very weak	2.84	Very, very weak
.50	" "	2.36	" "	2.71	" "	2.65	" "
2.37	" "			2.59	" "	2.59	" "
2.26	" "			2.43	Weak	2.45	" "
				2.26	"	2.26	" "
				2.18	"	2.19	" "

Essentially the same results were obtained by use of dextrans having a relative viscosity of 2.003 and 1.414. The pattern from the latter dextran is shown in Fig. 1, *a.*

Dextran; Drying of Aqueous Pastes—A viscous paste of dextran (relative viscosity[1] 2.003) was dried in two parts; one at 25°, the other at 115°. The former gave an amorphous pattern, the latter gave a poor quality x-ray line pattern of the type L-2 (see Fig. 1, *b*). A much thinner paste, when dried more slowly at 50°, produced an x-ray pattern of type L-1 of good quality.

Dextran; Products of Acid Hydrolysis—Dextran was hydrolyzed by 0.5

N sulfuric acid at 90° for 1, 2, and 4 hours, respectively, and the fractions of highest molecular weight were isolated from the hydrolysates. Complete details of these hydrolyses and fractionations will be given elsewhere.[2]

The fractions of highest molecular weight were separated from hydrolysis products of smaller size by dialysis of the sulfate-free solution and by repeated precipitation from aqueous solution by the addition of ethanol to make a 60 per cent ethanol concentration. During the fractional precipitations, the products from 2 and 4 hour hydrolyses became incompletely soluble in hot water. After the last fractional precipitation, the subfraction which had become insoluble in hot water was removed and found to give a good quality x-ray line pattern of type L-3. The main fraction was isolated from the clear supernatant in a manner analogous to that described for amorphous dextran (7).

These 60 per cent ethanol-insoluble fractions from 1 and 2 hour hydrolyses gave amorphous x-ray patterns and had $[\alpha]_D^{25} = +199°$ (in 1 N sodium hydroxide, $C = 1$) and about 65 and 40 glucose units, respectively, per reducing group. Ethoxyl analysis showed them to contain no ethanol. X-ray line patterns of the type L-3 and of very good quality were obtained after humidification or after treatment for 2 days with aqueous ethanol. Although humidification, which caused the amorphous samples to turn to cloudy pastes, appeared to make no significant change in the dried, powdery products from the treatment with aqueous ethanol, it caused these samples to produce intensified and sharper x-ray reflections.

Other fractions from 2 and 4 hour hydrolyses, the isolations of which are not described specifically here, have produced strong x-ray patterns, as is shown in Figs. 1 c and 1 d.

Dextrans Prepared by Other Investigators—X-ray examination of the dextran of Hassid and Barker[3] (1) and of the dextran from *Leuconostoc dextranicum*,[4] which is, presumably, the dextran referred to by Fairhead, Hunter, and Hibbert (13), gave results comparable to those for *Leuconostoc mesenteroides* dextran. In the states in which they were obtained, these samples produced amorphous x-ray patterns, but after treatment with 60 per cent ethanol the products gave x-ray line patterns of type L-1.

The dextran from *Leuconostoc dextranicum* dissolved readily to form aqueous pastes of very low viscosity. The dextran of Hassid and Barker, the dense, horny appearance of which was indicative of close molecular

packing and which has been described as water-insoluble (1), was obtained in 2 per cent aqueous solution by vigorous mechanical shaking. After precipitation of the dextran from this solution in the manner described for the preparation of amorphous dextran (7), the fluffy product was much more readily soluble in water than was the original sample.

Water-Insoluble Dextran—In the state in which they were isolated, both of the purified fractions of the water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523 (7) produced essentially amorphous x-ray patterns. Weak interferences were produced at 9.5 and 4.25 A by the fraction which was insoluble in the culture medium, and at 20.0 and 9.5 A by the fraction which was originally soluble in the culture medium but which became insoluble during purification. After exposure in 100 per cent relative humidity for 3 days at 25°, the first mentioned fraction produced a weak crystalline pattern which had an interference at 9.5 A, and the third through the ninth interferences of the dextran pattern, type L-3' (see Table I).

Each of these fractions appeared luminous under crossed Nicol prisms but no extinction was observed.

Amylaceous Carbohydrates—3 days humidification of corn amylose, which had been isolated from the amylose-butanol complex (14) as a chemically reactive, dry powder,[5] changed it to a coherent, elastic mass, and caused its x-ray pattern to change from a V to a B type. (When starch crystallizes in the presence of alcohol, usually a V pattern results; when it crystallizes in the presence of water alone, A or B patterns result.) Humidification of similarly prepared potato amylose resulted, after 3 days, in the replacement of its original well defined V pattern by an amorphous pattern. Continuing humidification for 3 weeks resulted in the development of a poor line pattern. Katz (9) observed that the V pattern of precipitated wheat starch was changed to the B pattern by humidification.

Methanol-extracted (15) glutinous corn-starch, contaminated with about 2 per cent ordinary corn-starch, was prepared in an amorphous state by precipitation of an aqueous paste in ethanol.[5] Humidification for 7 days caused the starch, which originally produced the amorphous x-ray pattern shown in Fig. 1, *e*, to produce the type A pattern shown in Fig. 1, *f*. Native granules of glutinous corn-starch are known to produce an x-ray line pattern (10).

A dry, amorphous sample of corn amylopectin, which had been treated with cotton to remove last traces of amylose (16), produced an A type starch pattern after 3 days humidification.

Glycogen has been reported to give only amorphous patterns under

[5] Jeanes, A., Deane, R. A., Whistler, R. L., and Hilbert, G. E., in preparation.

treatments which cause starch to give line patterns (17, 18). Application of the technique of treatment with aqueous ethanol as described for dextran resulted, even after 8 weeks under aqueous ethanol, in amorphous patterns only. Similarly, only amorphous patterns were obtained from the β-amylase limit dextrin of glutinous corn-starch.

Humidification caused both glycogen and β-amylase limit dextrin to turn to clear syrups, which, after drying, produced amorphous x-ray patterns.

Observations on Other Physical Properties

The behavior of samples under conditions of 100 per cent relative humidity at 25° not only reveals small differences in their ability to hydrate and dissolve but is also an indication of the x-ray pattern which the sample will give after this treatment. The variation of the humidified samples from rather dry, apparently unchanged powders through opaque to cloudy pastes to clear syrups directly paralleled the decreasing degree of orderly association, as indicated by the x-ray patterns.

Likewise, the order of solubility paralleled the physical condition of the sample. Thus, fluffy, amorphous dextran dissolved readily merely by exposure in an atmosphere saturated with water vapor at 25°, but the pulverized "glassy" product from a dextran paste dried at 115° dissolved slowly in cold water (20°), and the pulverized, "glassy" product from a paste dried slowly at 50° required heating to 70° to dissolve it in water. Fluffy, amorphous samples of acid-hydrolyzed dextrans dissolved readily in cold water, but the powdery crystalline products from the action of 60 per cent ethanol on these fractions as well as the pulverized, "glassy" crystalline products of humidification required heating in water at 100° to give clear solutions.

Formation of Filaments from Dextran—Filaments have been obtained from dextrans having a relative viscosity[1] of 1.855 and 2.003 and from "autolyzed" dextrans having a relative viscosity of 1.414 and 1.565. Aqueous pastes of the proper consistency for filament formation were obtained from "autolyzed" dextrans by 2 days humidification in air saturated with water vapor at 25°, but pastes obtained from the other dextrans under these conditions were too rubbery and viscous and had to be thinned by addition of water. When, under normal atmospheric conditions, a probe was touched to these pastes and then steadily pulled away, a lustrous filament was obtained. A filament many feet in length has been obtained by the use of a small mechanical reel. X-ray examination of a bundle of these fibers gave an amorphous pattern.

Filaments could not be obtained similarly from the dextran of Hassid and Barker[3] nor from glutinous corn-starch.

DISCUSSION

The emphasis that has been given to the amorphous nature of branched amylaceous substances in contrast to the crystalline nature of the linear substances has led recently to the erroneous belief that only the linear molecules can produce x-ray line patterns (19). In general, irregularity in molecular shape or structure is believed to interfere with orderly association (8). For example, the amorphous nature of the unbranched polysaccharide, lichenin, has been attributed to molecular irregularity resulting from the presence of both 1,3- and 1,4-glucosidic linkages (20). In amylaceous substances, branches are generally believed to provide the irregularity that interferes with crystallization. However, the view has been expressed that orderly association occurs between branches of sufficient length (21, 22) but not if the branches are modified or shortened (22). The results reported here on corn amylopectin, glutinous corn-starch, the β-amylase limit dextrin of glutinous corn-starch, and glycogen, in agreement with observations of Schoch and French (22), show that the tendency towards orderly molecular association decreases in the order stated; that is, in the order of increasing number or shortness of side chains (21, 23). However, the difference reported here in the behavior of potato as compared with corn amylose indicates that some still unrecognized factor can interfere with crystallization even in linear molecules which are apparently uniform in structure.

From the extremely careful methylation study of Levi, Hawkins, and Hibbert on a water-soluble dextran from *Leuconostoc mesenteroides* (2), it appears that this substance has greater structural irregularity and more numerous as well as shorter branches than glycogen. In view of this and of the influential rôle that branching seems to play in retarding crystallization in amylaceous polysaccharides, the observation of crystallinity in dextran seems to be anomalous. It is possible that crystallization might be more favored in dextran than in glycogen by the predominance of glucosidic linkages involving a primary position on carbon 6. It is also possible that the concept of the structure of dextran is inaccurate and that clarification may have to await further structural information on dextrans of specific origin.

Dextran produces a series of typical x-ray diffraction powder patterns which are entirely different from the patterns of starch. The relative intensities of the lines and the background in patterns from the high molecular weight dextrans indicate that the amount of crystalline material present is rather small. A treatment which resulted in crystallization of the high molecular weight dextran from *Leuconostoc mesenteroides* NRRL B-512 also resulted in about the same degree of crystallinity in two other dextrans

from widely different sources. This is interpreted to indicate that the x-ray line pattern common to these three dextrans is due to a structure which is characteristic of dextran rather than to the presence of a small fraction of material with exceptional crystallizing ability.

The fractions of highest molecular weight from 1, 2, and 4 hour acid hydrolyses of dextran progressively developed crystallinity more readily and completely than dextran. This might result from random reduction of molecular size or from increased regularity in size or structure of the molecules during acid hydrolysis. Increased regularity in structure is to be expected from preferential hydrolysis of the α-1,4-glucosidic linkages, which we have found to have a critical increment 3000 calories per mole less than that of α-1,6-linkages.[2]

Numerous water-insoluble polysaccharides such as amylose (14), cellulose (19), pectins (24), and some of the fractions produced by *Leuconostoc mesenteroides* NRRL B-512 (7), or obtained from its water-soluble dextran by acid hydrolysis, produce strong x-ray line patterns. Unlike all these other substances, the dextran from *Leuconostoc mesenteroides* NRRL B-523 gave an amorphous x-ray diffraction pattern, was not horny, and yet was water-insoluble. Molecular association in this dextran must be extensive but arrangement of the associated chain segments in a 3 dimensional periodic pattern is lacking.

Investigations on cellulose esters (25) and proteins (26) have established that water, or water and heat, facilitate motion and crystallization of chain segments of these substances in the solid state. Similar observations have been made on starch granules (27) and on starch coacervates (22). Our observations provide further examples of this principle. Exposure of solid samples in an atmosphere saturated with water vapor at 25° caused some amorphous substances to assume a crystalline state, crystalline substances to change from one modification to another or develop sharper patterns, and one substance passed through an amorphous state during its transition from one crystalline state to another. Crystallization resulted when a dextran paste was warmed while being dried slowly, but did not result when the slow drying was at 25°. Hydrated masses of dextran developed crystallinity when allowed to stand under specific optimum concentrations of aqueous ethanol, presumably because a critical state of dehydration was obtained which was favorable to molecular orientation and association.

SUMMARY

1. Orderly molecular association has been demonstrated by x-ray analysis in branched chain polysaccharides including water-soluble dextran from *Leuconostoc mesenteroides* NRRL B-512, corn amylopectin, and glu-

DISCUSSION

The emphasis that has been given to the amorphous nature of branched amylaceous substances in contrast to the crystalline nature of the linear substances has led recently to the erroneous belief that only the linear molecules can produce x-ray line patterns (19). In general, irregularity in molecular shape or structure is believed to interfere with orderly association (8). For example, the amorphous nature of the unbranched polysaccharide, lichenin, has been attributed to molecular irregularity resulting from the presence of both 1,3- and 1,4-glucosidic linkages (20). In amylaceous substances, branches are generally believed to provide the irregularity that interferes with crystallization. However, the view has been expressed that orderly association occurs between branches of sufficient length (21, 22) but not if the branches are modified or shortened (22). The results reported here on corn amylopectin, glutinous corn-starch, the β-amylase limit dextrin of glutinous corn-starch, and glycogen, in agreement with observations of Schoch and French (22), show that the tendency towards orderly molecular association decreases in the order stated; that is, in the order of increasing number or shortness of side chains (21, 23). However, the difference reported here in the behavior of potato as compared with corn amylose indicates that some still unrecognized factor can interfere with crystallization even in linear molecules which are apparently uniform in structure.

From the extremely careful methylation study of Levi, Hawkins, and Hibbert on a water-soluble dextran from *Leuconostoc mesenteroides* (2), it appears that this substance has greater structural irregularity and more numerous as well as shorter branches than glycogen. In view of this and of the influential rôle that branching seems to play in retarding crystallization in amylaceous polysaccharides, the observation of crystallinity in dextran seems to be anomalous. It is possible that crystallization might be more favored in dextran than in glycogen by the predominance of glucosidic linkages involving a primary position on carbon 6. It is also possible that the concept of the structure of dextran is inaccurate and that clarification may have to await further structural information on dextrans of specific origin.

Dextran produces a series of typical x-ray diffraction powder patterns which are entirely different from the patterns of starch. The relative intensities of the lines and the background in patterns from the high molecular weight dextrans indicate that the amount of crystalline material present is rather small. A treatment which resulted in crystallization of the high molecular weight dextran from *Leuconostoc mesenteroides* NRRL B-512 also resulted in about the same degree of crystallinity in two other dextrans

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EXPLANATION OF PLATE

Plate 3

Fig. 1. X-ray diffraction patterns of dextran, acid-hydrolyzed dextrans, and glutinous corn-starch.   *a*, type L-1 from dextran of relative viscosity 1.414 after treatment with 60 per cent ethanol followed by humidification; *b*, type L-2 from a dextran paste dried at 115°; *c*, dextran type L-3 from a fraction of 4 hour acid hydrolysis after treatment with 70 per cent ethanol followed by humidification; *d*, dextran type L-3′ from a fraction of 2 hour acid hydrolysis after humidification; *e*, pattern from amorphous glutinous corn-starch; *f*, type A starch pattern from amorphous glutinous corn-starch after humidification.
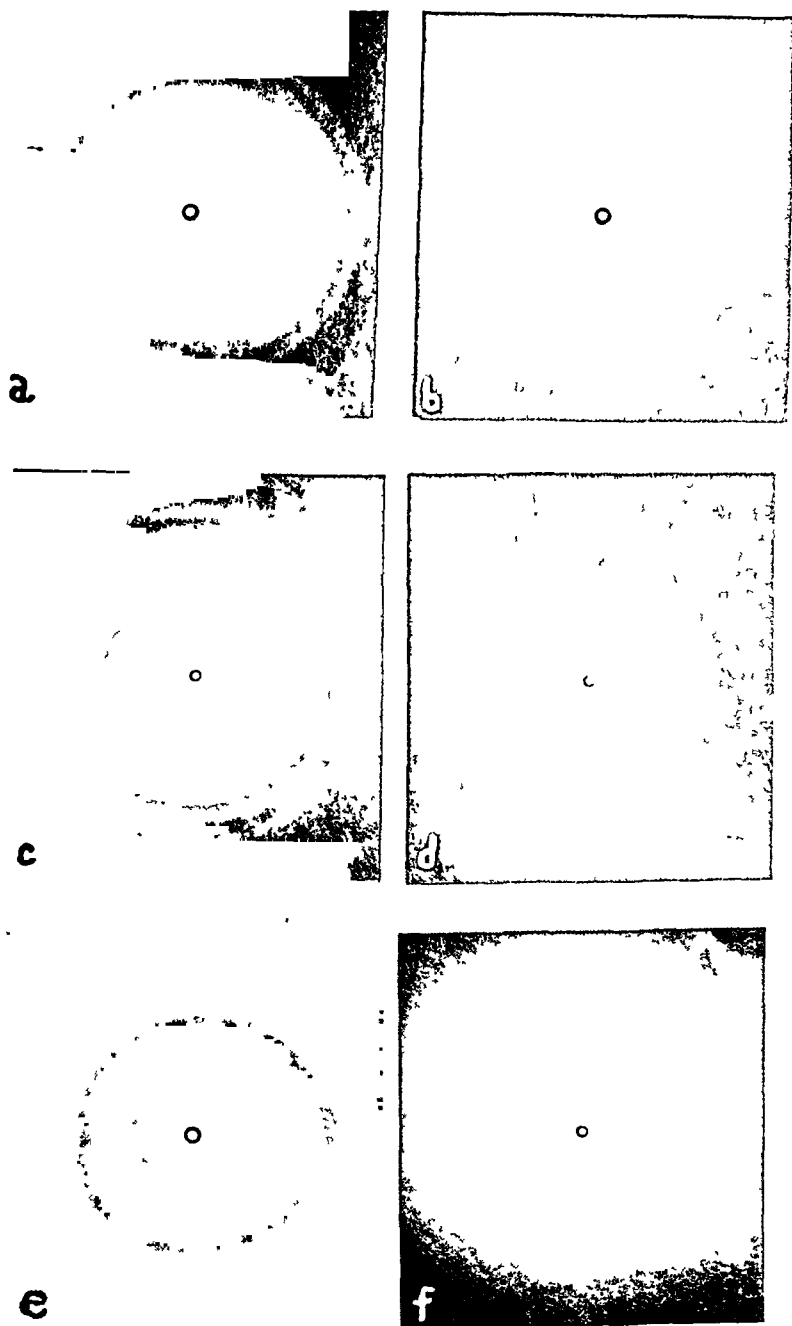
(Jeanes, Scheltz, and Wilham   Dextran and amylaceous carbohydrates)

FUNCTION OF THE VITAMIN B$_6$ GROUP: MECHANISM OF TRANSAMINATION

By W. W. UMBREIT,* D. J. O'KANE,† and I. C. GUNSALUS‡

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The rôle of vitamin B$_6$ in amino acid metabolism has been well established through enzyme studies (1–3) and by the substitution for various amino acids in growth studies (4–6).

The coenzyme form, synthetic pyridoxal phosphate (7), functions as the coenzyme for amino acid decarboxylases (8–10), transaminases (2, 3), tryptophan formation (11), and tryptophan breakdown by tryptophanase (12). The reactions involved in the amino acid replacement of vitamin B$_6$ have not been determined.

The mechanism by which pyridoxal phosphate functions is still in question. Snell (13), however, found that pyridoxal and pyridoxamine were readily and reversibly interconverted by *in vitro* transamination and suggested on this basis that biological transamination might be mediated by the transfer of the amino group via the aldehyde and amino forms of vitamin B$_6$ to the keto acid. The demonstration of pyridoxal phosphate as the coenzyme of transamination quite naturally suggested the existence of pyridoxamine phosphate and its possible rôle in those systems for which pyridoxal phosphate is the coenzyme.

Umbreit, O'Kane, and Gunsalus (14) found activation of the apotransaminase of dried bacteria by "pyridoxamine phosphate" which had been prepared by the method of Snell for the interconversion of pyridoxal and pyridoxamine (13). The pyridoxamine phosphate was judged to be free of pyridoxal phosphate by its lack of coenzyme activity for tyrosine apodecarboxylase and by the spectrum. Later Ames, Sarma, and Elvehjem (15) found pyridoxamine phosphate, prepared as above to stimulate the transaminase in liver homogenates from vitamin B$_6$-deficient animals. These data could be interpreted as substantiating the hypothesis of pyridoxamine phosphate as an intermediate in transamination, although the alternative possibility of the transformation of the pyridoxamine phosphate to pyridoxal phosphate by a separate enzyme was not excluded. The latter type

of transformation occurs in *Streptococcus faecalis*, as indicated by the function of pyridoxamine for tyrosine apodecarboxylase only after incubation with cells and pyruvate (16).

A critical test of the mechanism of transamination and the possible rôle of the two coenzyme forms as intermediates would require a purified apoenzyme in which the multiplicity of side reactions, possible in dried bacterial cells or homogenates, was eliminated.   For this purpose the glutamic-aspartic transaminase of pig heart was resolved and purified (17).   On test the pyridoxamine phosphate preparations were not active, whereas the pyridoxal phosphate activated the enzyme.   The pyridoxamine phosphate was, as mentioned previously, also inactive for tyrosine apodecarboxylase and for tryptophanase.   In addition to the enzyme data, the spectra of pyridoxal and pyridoxamine and their respective phosphates, by which all four may be differentiated, are reported.   Recently, Snell *et al.* have reported differential analysis of the four by growth assays (18, 19).

Methods

Enzyme—The enzymes were prepared as previously reported from this laboratory: the tyrosine decarboxylase from cells grown in vitamin B₆-deficient medium and dried *in vacuo* (20); the bacterial apotransaminase by similar process, except that the cells were grown in a neutral medium (2); the purified glutamic-aspartic apotransaminase by resolution of the enzyme from pig heart (17) and its purification by the method of Green (21).

Coenzymes—Synthetic barium pyridoxal phosphate (7) was used.   Pyridoxamine phosphate was not synthesized directly, but was prepared by treating pyridoxal phosphate with glutamic acid and heat according to the method of Snell (13) for the transformation of pyridoxal to pyridoxamine. No inorganic phosphate was released by the heating and pyridoxal phosphate was absent as shown by the enzymatic test with tyrosine apodecarboxylase and by the spectrum, as indicated below.   The reaction could also be reversed, although not completely, by treating the pyridoxamine phosphate samples with α-ketoglutarate, again according to Snell's method. The preparation of pyridoxamine phosphate in pure form free from pyridoxal phosphate and its chemical characterization is essential for the proof of mechanism of the reactions studied.

Spectra of Pyridoxal, Pyridoxamine, and Their Phosphates

Pyridoxal, in contrast to other members of the vitamin B₆ group, exhibits a yellow color at alkaline reaction with an absorption maximum at 385 mμ.   This should not be confused with the ultraviolet spectrum which is

possessed by all members of the group. The intensity of the absorption is increased in pyridoxal phosphate, as compared to pyridoxal, without appreciable shift in the wave-length of the band. The spectra for these two compounds at various pH levels are shown in Fig. 1. Especially to be
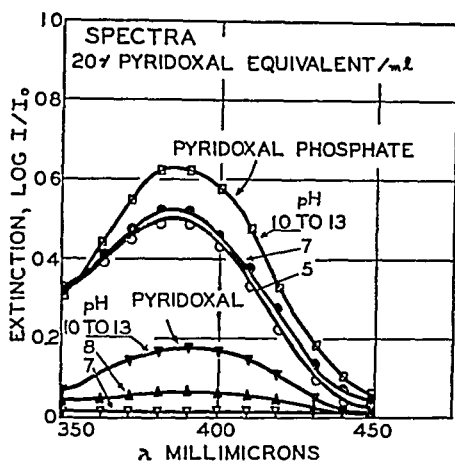
FIG. 1. Effect of pH on absorption spectra of pyridoxal and pyridoxal phosphate
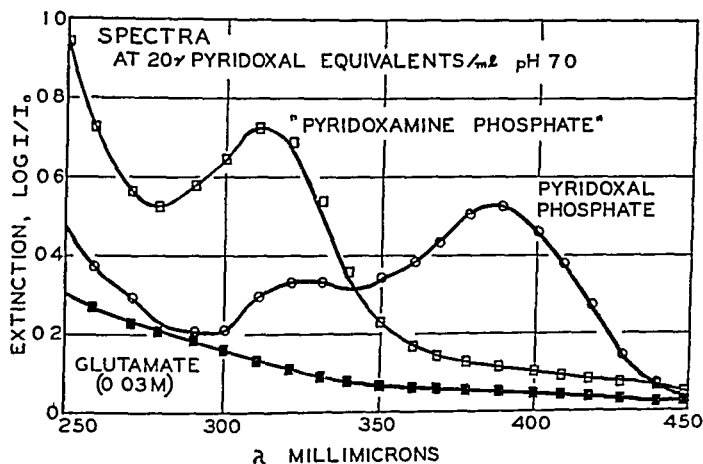
FIG. 2 Spectra of pyridoxal phosphate and pyridoxamine phosphate   Pyridoxamine phosphate obtained by heating pyridoxal phosphate (100 γ per ml) at 121° for 30 minutes with 0 03 M glutamate, pH 7 0

noted is the absorption at pH 7; *i.e.*, the pyridoxal absorption is nil, whereas the pyridoxal phosphate absorption is more than 90 per cent maximum.

If one heats pyridoxal phosphate with glutamic acid (30 minutes, 121°), the absorption at 385 mμ disappears with the formation of pyridoxamine

phosphate. The spectra of these compounds, in the range 250 to 450 mµ at pH 7, are shown in Fig. 2. Incubation of pyridoxal phosphate with glutamic acid or with glutamic-aspartic transaminase results in a slight decrease in absorption at 385 mµ and a shift of the maximum toward the
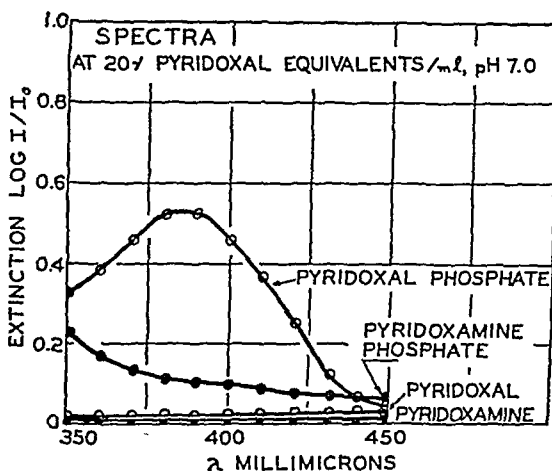
FIG. 3. Differentiation of pyridoxal and pyridoxamine from their phosphates by spectrum.
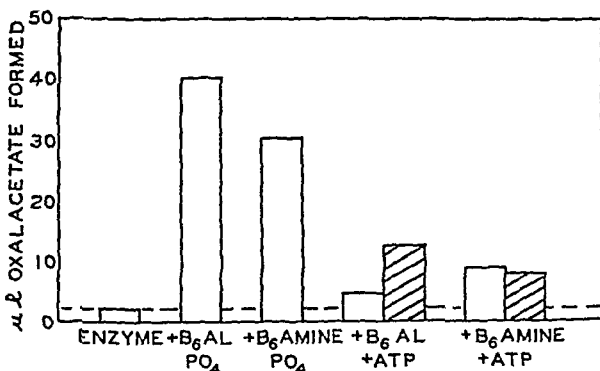
FIG. 4. Activation of apotransaminase of vitamin B₆-deficient *Streptococcus faecalis*.

longer wave-lengths. This may possibly indicate a coordination of the amino groups with the free aldehyde of pyridoxal.

The absorption spectra for free pyridoxal and pyridoxamine and their phosphates at pH 7 are shown in Fig. 3. Thus the pyridoxal and pyridoxamine phosphates can be distinguished, and each distinguished from its non-phosphorylated analogue. Pyridoxal may be differentiated from pyridoxamine by the increased absorption at alkaline reaction (Fig. 1).
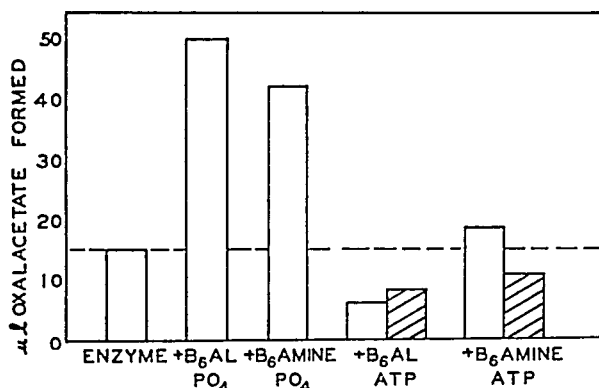
FIG. 5. Activation of cell-free resolved bacterial apotransaminase with pyridoxal and pyridoxamine phosphate.

TABLE I

Pyridoxal Phosphate and Pyridoxamine Phosphate Activation of Transaminase Streptococcus faccalis R, Dried Cells

Per Warburg cup, 0.5 ml. of 0.2 M phosphate buffer, pH 6.5, enzyme, coenzyme, water to 1.9 ml.; 1st side arm, 0.5 ml. of 0.4 M aspartic acid, 0.3 ml. of 0.2 M α-ketoglutaric acid; 2nd side arm, 0.5 ml. of aniline citrate.

	Microliters oxalacetate formed per 30 min.				
Sample, treatment	None	Pyridoxal phosphate	Pyridoxamine phosphate*	Pyridoxal ATP	Pyridoxamine ATP
		per 10 γ	*per 10 γ*	*per 10 γ per 1 mg.*	*per 10 γ per 1 mg.*
T₄, autolyzed 18 hrs., 37° 0.1 M phosphate, pH 7.5, supernatant dialyzed	14	50	41	7	10
R₆T, autolyzed 18 hrs., 37°, 0.1 M phosphate, pH 7.3, supernatant used immediately	14	58	73	16	19
Run†					
T₄, 7 days	18	61	35		
T₅	2	37	34		
R₆T₂, 4 days	15	102	30		
" 7 "	23	97	36		
" 31 "	7	127	11		
1608, 7 "	14	43	56		
Acetone-dried pig heart (12 days)	81	126	78		

* 265 to 530 mγ without activity with tyrosine decarboxylase; the same quantity of pyridoxal phosphate gave full activity.

† Aspartic acid added to the cup, α-ketoglutarate tipped after 10 minutes. All the samples autolyzed 18 hours, 37°, and allowed to stand 5° in 0.1 M phosphate buffer, pH 7.5, for the days indicated.

Cotransaminase Activity of Pyridoxamine Phosphate

Dried Bacterial Cells—The glutamic-aspartic apotransaminase of *Streptococcus faecalis* R was prepared by two methods, first by the growth of vitamin B_6-deficient cells and second by resolution of the enzyme from cells grown in a complete medium, as described by Lichstein, Gunsalus, and Umbreit (2).

The apoenzyme from deficient cells was activated by pyridoxal or pyridoxamine phosphate to about an equal extent (Fig. 4), and to a lesser degree by pyridoxal or pyridoxamine in the presence of adenosine triphosphate (ATP). The cell-free enzyme prepared from dried cells and resolved

TABLE II

Pyridoxal Phosphate and Pyridoxamine Phosphate with Purified Pig Heart Transaminase

Per Warburg cup, 0.5 ml. of 0.2 M phosphate, pH 7.3, 0.1 ml. of enzyme, coenzyme, and water to 1.7 ml.; 1st side arm, 0.25 ml. of 0.8 M aspartic acid,* 0.25 ml. of 0.4 M α-ketoglutarate; 2nd side arm, 0.5 ml. of aniline citrate.

Enzyme	Additions	Microliters oxalacetate formed per 10 min., 37°		
		None	Pyridoxal phosphate	Pyridoxamine phosphate†
			per 10 γ	*per 10 γ*
Sample 0, diluted 1:40‡	Incubate with asparate	4	91	12
	" " α-ketoglutarate	4	56	9
			per 1 γ per 5 γ	*per 1 γ per 5 γ*
	Tip both	4	13 36	9 11

* Add one to the cup, tip the other from the side arm after 10 minutes.

† 500 mγ inactive in tyrosine decarboxylase, 50 mγ of pyridoxal phosphate give full activity.

‡ We wish to thank Doreen O'Kane for this enzyme sample; see (17).

was also activated by pyridoxal or pyridoxamine phosphate, though not by the unphosphorylated compounds even in the presence of ATP (Fig. 5). These data were interpreted as indicating the function of pyridoxamine phosphate as a coenzyme for transamination, in addition to pyridoxal phosphate as previously reported (1, 2), and thus furnished evidence in favor of Snell's hypothesis for the mechanism of coenzyme action (13).

In order to generalize upon the evidence for the intermediate function of pyridoxamine phosphate, five separate *Streptococcus faecalis* R cell preparations were tested (Table I). When tested as partially resolved cell-free preparations, four of these were activated, whereas the fifth, Sample R_6T_2, was not. Also, as shown in Table I, a sample of acetone-dried pig

heart was partially resolved and could be activated by pyridoxal phosphate but not by pyridoxamine phosphate.

The latter two observations cast serious doubt upon the direct mediation of pyridoxamine phosphate in transamination, since, if this substance were acting by direct mechanism, all cases should be positive.

Resolved Pig Heart Transaminase—To test critically the activity of pyridoxamine phosphate in transamination, it was felt that a purified enzyme, free of possible supplementary enzymes which catalyzed various side reactions, would be highly desirable. Therefore, the glutamic-aspartic transaminase of pig heart was resolved and purified (17). As shown in Table II, pyridoxal phosphate, but not pyridoxamine phosphate, was active with this enzyme. These data, in addition to the data with partially resolved acetone-dried pig heart enzyme, constitute evidence against pyridoxamine phosphate as a coenzyme of transamination. It should be recalled, however, that these data are subject to the limitation of chemical criteria for the identity of the pyridoxamine phosphate and must, therefore, remain tentative.[1]

DISCUSSION

The activity of pyridoxamine phosphate, prepared by heating pyridoxal phosphate with glutamic acid, for the glutamic-aspartic transaminase of dried bacterial cells and the lack of activity for the purified pig heart enzyme leave the evidence for the mechanism of coenzyme action in an anomalous state. While the extension of the data to several dried bacterial preparations strengthens the possibility, the single exception (Sample R_6T_2, Table I) constitutes stronger evidence against this mechanism.

Contradictory data from two sources constitute lack of substantiation of the pyridoxal phosphate-pyridoxamine phosphate mechanism and suggest that the Schlenk and Fisher (22) interpretation of the preliminary experiments of Umbreit, O'Kane, and Gunsalus (14) as conclusive evidence may be premature.

At present the following possibilities as to the disagreement in the data exist. (1) The proposed mechanism is incorrect. (2) The mechanism for the bacterial enzyme and pig heart enzyme is different; this seems highly unlikely. (3) The preparation obtained by heating glutamic acid with pyridoxal phosphate is not pyridoxamine phosphate, but is a compound which the bacterial cells can convert to an active compound, either to pyridoxamine phosphate or to pyridoxal phosphate.[1] Pyridoxamine phos-

[1] Snell (personal communication) has now shown growth-promoting properties of pyridoxamine phosphate produced by heating pyridoxal phosphate with glutamic acid to coincide with those of the compound prepared in the Research Laboratories of Merck and Company by the direct phosphorylation of pyridoxamine.

phate, as such, is not active in the transaminase reaction, but may be converted into pyridoxal phosphate by supplementary enzymes present in the bacterial preparations. An analogy for this is found in the formation of coenzyme for tyrosine decarboxylase by resting suspensions of *Streptococcus faecalis* in the presence of pyridoxamine, provided that the cells are incubated with a keto acid before the decarboxylase activity is determined.

The demonstration of Snell *et al.* (18, 19) by microbiological assay of a compound in natural material possessing the properties of pyridoxamine phosphate and its replacement by material prepared by heating pyridoxal phosphate with glutamic acid clarifies the natural occurrence of pyridoxamine phosphate and strengthens the evidence for the nature of the heated product used with the bacterial cell-free transaminase.

SUMMARY

Preparations of "pyridoxamine phosphate" obtained by heating pyridoxal phosphate with glutamic acid under the conditions suggested by Snell for the formation of pyridoxamine from pyridoxal yield preparations which are active in stimulating the glutamic-aspartic transaminase enzyme of dried cells of *Streptococcus faecalis* R.

These pyridoxamine phosphate preparations are not active with tyrosine decarboxylase, nor with purified glutamic-aspartic transaminase apoenzyme of pig heart, both of which are activated by pyridoxal phosphate.

The results of these findings are discussed and their implications pointed out.

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THE NATURE OF THE CIRCULATING THYROID HORMONE*

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Iodine exists in the thyroid gland in at least two well defined chemical entities, namely diiodotyrosine and thyroxine, which, according to Harington, account for practically all of the organic iodine in the gland ((2) p. 91). These two compounds are not present in a free form but are combined with other amino acids to form the characteristic thyroid protein, thyroglobulin.

Iodine is also a component of plasma, but its concentration there is so minute (about 5 γ per 100 cc.) that the chemical form in which it exists has eluded discovery. It was formerly believed that plasma iodine is also present as thyroglobulin (3, 4), but this view has been abandoned, mainly as a result of the studies of Trevorrow (5) and of Lerman (6). Despite the fact that crystalline thyroxine, when administered, produces in the mammalian organism all the known effects of thyroid tissue, investigators have nevertheless hesitated to assert that the circulating form of the thyroid hormone is thyroxine *per se*. This rejection is based on the following observations: (1) the failure of some investigators to account completely for the biological activity of thyroglobulin by its thyroxine content ((7, 8), ((9) p. 114), (2) the delayed response of animals to injected thyroxine ((2) p. 123, (10)), and (3) the failure of thyroxine to act *in vitro* (11, 12). These observations led Harington in 1935 (8, 13) to postulate that the circulating hormone is a peptide containing both thyroxine and diiodotyrosine. Recently, however, reevaluating this older evidence, Harington (14) has arrived at the conclusion that the peptide concept is an unnecessary complication and that thyroxine itself is probably the circulating hormone.

Two powerful tools, namely a refined method for determining small quantities of iodine (15) and the radioactive isotope of iodine (I^{131}), made possible a new attack on the problem of the nature of plasma iodine. The evidence provided by these means is presented here.

EXPERIMENTAL

The experiments presented here may be grouped conveniently as follows: (1) those dealing with the extractability of plasma iodine with butyl al-

cohol; (2) those dealing with the butyl alcohol extractability of thyroxine added to plasma; (3) the demonstration that the thyroid hormone of plasma labeled by means of radioactive iodine behaves exactly the same as added thyroxine carrier, as judged by the latter's recrystallization to constant specific activity and by its distribution between two immiscible solvents; (4) those dealing with the combination of thyroxine with plasma protein.

Extraction of Plasma Iodine with Butyl Alcohol

The organic solvent n-butyl alcohol has proved helpful in the determination of the thyroxine content of thyroid tissue. It was first used for this purpose in 1932 by Leland and Foster (16), who showed that it extracted all of the thyroxine from thyroid protein that had been subjected to strong hydrolysis with 2 N NaOH. Later several investigators (5, 17–19) used butyl alcohol for the fractionation of blood iodine, but their efforts led to no agreement as to the nature of the circulating thyroid hormone. This is not surprising in view of the difficulties encountered in measuring the small quantities of iodine involved in such experiments.

A sensitive and reliable method for the determination of plasma iodine, previously reported from this laboratory (15), enabled us to carry out butyl alcohol fractionation on smaller quantities of plasma than was previously possible and thus to achieve complete extraction with convenient volumes of solvent. The details of this procedure are described below.

3 cc. of heparinized plasma were added, with shaking, to 15 cc. of normal butyl alcohol (reagent grade) in a 50 cc. narrow necked centrifuge tube. The tube was stoppered (the rubber stopper had previously been treated with alkali and acid and then soaked in butyl alcohol) and shaken thoroughly. The butyl alcohol layer obtained by 10 minutes of centrifugation was quite clear and was transferred to a 125 cc. separatory funnel. The residue was reextracted twice, each time with 15 cc. of butyl alcohol, and the mixture centrifuged after each addition. The three clear butyl alcohol extracts were combined in the separatory funnel and shaken with 50 cc. of a reagent consisting of 4 N NaOH and 5 per cent Na_2CO_3. The latter reagent, introduced by Blau (20), extracts inorganic iodide and diiodotyrosine, but not thyroxine, from butyl alcohol. After the mixture was allowed to stand for a few hours, the lower aqueous layer was run out and the butyl alcohol fraction extracted a second time with 30 cc. of 4 N NaOH-5 per cent Na_2CO_3. This time the separation of the two layers was allowed to proceed for 15 hours, at the end of which time the butyl alcohol layer showed only a slight turbidity. The butyl alcohol was next transferred to the two-neck digestion flask used in the iodine determination and carefully concentrated to dryness under reduced pressure at 100°. The iodine deter-

mination was carried out on the residue as previously described by us for plasma iodine (15) except for the following modifications: (1) Hydrogen peroxide (1 cc. of a 1:20 dilution of Merck's superoxol) was added after the addition of the phosphorous acid (21). The slight color in the distillate previously encountered when the peroxide was added (15) did not appear in the present investigation. We attribute this to the more careful construction of the iodine still used in this later work. (2) It seemed desirable to add some non-iodine-containing organic material to the butyl alcohol residue in order to make it more comparable to the original plasma. For this purpose we used dried defatted muscle containing a minimum amount of iodine. Such muscle was obtained from rats that had been fed a low iodine diet containing 0.15 per cent propylthiouracil. In some of the samples wheat was added as organic carrier, since it was found in control runs that it gave exactly the same results as the dried muscle. Two samples of butyl alcohol were shaken with the alkali reagent and concentrated to dryness as described above; these served as reagent blanks.

The results obtained by this butyl alcohol fractionation are shown in Table I. Blood was obtained from normal human subjects; a sufficient quantity was removed from each to permit carrying out triplicate or quadruplicate analyses on each plasma sample. The total and protein-bound iodine content of plasma was also determined for each subject. The protein-bound fraction was determined on a zinc hydroxide precipitate which had been washed twice with redistilled water.

It is clear from Table I that 90 per cent or more of the iodine in plasma is extractable with butyl alcohol at room temperature. This may be taken to mean that the iodine in plasma is not stably bound to protein. These results should be contrasted with those in Table II, which shows the extent to which the iodine of fresh thyroid tissue can be extracted with butyl alcohol; only a small per cent was found soluble. Only after strong hydrolysis does thyroid iodine become markedly soluble in butyl alcohol. There can be little doubt therefore that the chemical form of the iodine in plasma is different from that in the thyroid gland and that some degradation of the thyroid protein molecule takes place before the hormone is secreted into plasma.

As is shown in Table I, some iodine can be reextracted from the butyl alcohol by shaking the latter with the 4 N NaOH-5 per cent Na_2CO_3 reagent. This iodine, amounting to about 10 to 15 per cent of that present in the butyl alcohol, should represent the combined inorganic iodide and diiodotyrosine contents of the extract. Thus the diiodotyrosine content of plasma is at most only about 10 per cent of total plasma iodine. In the gland, however, diiodotyrosine iodine represents at least 60 per cent of the

total iodine (22). It is apparent therefore that there is a preferential release of thyroxine by the gland into the circulation; only a small fraction of the gland's iodine leaves the gland as diiodotyrosine.

TABLE I

Extractability of Plasma Iodine with Butyl Alcohol

Plasma source	Iodine in 3 cc. plasma					
	Total	Protein-bound	Butyl alcohol-extractable		Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na₂CO₃	
	γ	γ	γ	per cent of total	γ	per cent of total
Human female	0.188	0.183	0.174	93	0.150	80
" male	0.165	0.165	0.162	98	0.135	82
" "	0.168	0.174	0.147	88	0.156	93
" "	0.195	0.198			0.158	81
" "	0.162	0.156	0.153	94	0.126	78
" "	0.153	0.150			0.123	82
" "	0.168	0.156	0.162	96	0.123	73
Rat, pooled	0.094	0.096	0.093	99	0.081	84

TABLE II

Extractability of Rat Thyroid Iodine with Butyl Alcohol

Thyroids of ten large rats were pooled, minced with scissors, and duplicate portions taken for the following treatment.

Total I in gland	Trichloroacetic acid-soluble I*	Butyl alcohol-extractable I†	Butyl alcohol-extractable, not reextractable with 4 N NaOH-5 per cent Na₂CO₃
mg. per cent	per cent of total	per cent of total	per cent of total
17.3	6.9	3.4	2.4

* Thyroid tissue was homogenized with 1 cc. of 10 per cent trichloroacetic acid in a small glass tube.

† Thyroid tissue was homogenized with 1 cc. of butyl alcohol in a small glass tube. The residue was then extracted three times, each time with 3 cc. of butyl alcohol.

Behavior of Thyroxine Added to Plasma

The finding that approximately 80 per cent of the iodine in plasma can be extracted with butyl alcohol from which it cannot be reextracted with the 4 N NaOH-5 per cent Na₂CO₃ reagent suggested that this iodine is thyroxine. It therefore became of interest to compare the properties of this iodine fraction of plasma with those of crystalline thyroxine.

Butyl Alcohol Extraction—Crystalline thyroxine was added to human

plasma in an amount comparable to that already present, and this treated plasma was subjected to the butyl alcohol extraction procedure described above. Approximately 80 per cent of the added thyroxine was recovered in the final butyl alcohol extract (Table III). This compares favorably with the solubility properties of the iodine originally present.

Protein Precipitation—From the results in Table I it is clear that practically all of the iodine in plasma precipitates with proteins when zinc hydroxide is used as the precipitating agent. When crystalline thyroxine is added to plasma, it too precipitates quantitatively with proteins, as shown in Table IV. These results are in accord with those reported previously by Trevorrow (5) and by Bruger and Member (23).

TABLE III

Butyl Alcohol Extractability of Crystalline Thyroxine Added to Human Plasma

Procedure	Iodine determined in 3 cc plasma		Thyroxine I added per 3 cc plasma*	Thyroxine I recovered	
	Initial value	After adding thyroxine			
	γ	γ	γ	γ	per cent of that added
Total iodine	0.168	0.336	0.168		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.123	0.252		0.129	77
Total iodine	0.174	0.393	0 219		
Extractable with butyl alcohol but not reextractable with 4 N NaOH-5% Na_2CO_3	0.129	0.300		0 171	78

* The difference between the third and second columns.

Dialysis—The iodine in plasma is not dialyzable, as is shown in Table V and as reported previously by Silver and Tyson (24). A small amount of thyroxine when added to plasma is not dialyzable, although in simple aqueous solution thyroxine will dialyze readily.

Extraction of Diiodotyrosine from Plasma to Which It Had Been Added—Crystalline diiodotyrosine was added to plasma and the mixture extracted with butyl alcohol as described above. The amount of iodine added as diiodotyrosine was approximately equal to the total iodine initially present in the plasma sample. Table VI shows that the addition of 5 to 6 γ per cent of diiodotyrosine iodine to plasma did not affect the value obtained for the iodine in the thyroxine fraction. The method described here, therefore, should prove valuable in distinguishing between thyroxine iodine and diiodotyrosine iodine in plasma.

Unfortunately, the presence in plasma of 17 γ per cent of the gallbladder

dye, tetraiodophenolphthalein, did affect the thyroxine iodine value appreciably. The method described here is therefore not suitable for the determination of hormonal iodine in patients in which visualization tests of the gallbladder were recently carried out with this organic iodine compound.

It may be concluded from the experiments described in this section that crystalline thyroxine added to plasma behaves almost exactly like the

TABLE IV

Precipitation of Added Thyroxine Iodine with Plasma Proteins When Zinc Hydroxide Was Used As Precipitating Agent

Plasma source	Iodine initially present in 3 cc. plasma		Thyroxine I added	Iodine present in protein ppt.	Recovery of added thyroxine in protein ppt.
	Total	Protein-bound			
	γ	γ	γ	γ	*per cent*
Human........	0.141	0.138	0.234	0.363	96
"	0.168	0.156	0.168	0.336	107
Dog......................	0.14		2.08	2.16	97

TABLE V

Dialysis of Thyroxine Added to Human Plasma

Iodine initially present in 3 cc. plasma		Thyroxine I added	Non-dialyzable I	Recovery of non-dialyzable thyroxine I	
Total	Protein-bound				
γ	γ	γ	γ	γ	*per cent of added thyroxine*
	0.147	0	0.156		
0.168	0.156	0	0.168		
0.141	0.138	0.234	0.367	0.229	98
0.168	0.156	0.168	0.327	0.171	102

naturally occurring iodine of plasma. Such experiments suggest very strongly that the iodine in plasma is mainly in the form of thyroxine loosely attached to plasma protein. More evidence on this point was obtained with the aid of radioactive iodine, to be described in the following section.

Experiments with Radioactive Iodine

It has been shown in this laboratory that, after the injection of a carrier-free dose of radioiodine into rats that have been fed a low iodine diet, the isotope is rapidly taken up by the thyroid gland, converted there to organic iodine, and then released into the plasma (25). Within 24 hours, about 90 per cent of the radioactive iodine in plasma is protein-bound and nearly

all of this percentage is presumably the thyroid hormone. This procedure thus permits one to obtain *labeled* thyroid hormone in its normal physiological state.

Butyl Alchol Extraction of Radioactive Iodine from Rat Plasma—Twelve large rats were injected with 80 microcuries of I^{131} and their blood removed 24 hours later and pooled. The plasma was separated, and 3 cc. portions were taken for determination of total iodine, protein-bound iodine, and

TABLE VI

Effect of Presence of Diiodotyrosine and Tetraiodophenolphthalein on Determination of Thyroxine Iodine Content of Human Plasma

Substance added	Iodine added per 3 cc plasma	Iodine in butyl alcohol fraction prepared from 3 cc. of plasma	
		Original plasma	Plasma with added I
	γ	γ	γ
Diiodotyrosine	0 16	0 129	0 123
"	0 17	0 123	0.123
Tetraiodophenolphthalein*	0 51	0 123	0 345

* Trade name, Iodeikon.

TABLE VII

Extraction of Chemical and Radioactive Iodine from Plasma of Rats Injected 24 Hours Previously with Radioactive Iodine (I^{131})

	Iodine in 3 cc plasma		Per cent of total I	
	Chemical I	Radioactive I	Chemical I	Radioactive I
	γ	*counts per sec*		
Total I	0 096	348		
Protein-bound I	0 096	321	100	92
Butyl alcohol-extractable	0 093	250	97	72
" " but not icex-tractable with 4 N NaOH-5% Na_2CO_3	0 081	230	84	66

butyl alcohol-extractable iodine (Table VII). Both chemical and radioactive measurements were made on all plasma samples. Since the chemical method involved the distillation of the iodine into an alkaline medium, a portion of this alkaline distillate was used for the radioactive measurement by simply evaporating a suitable aliquot in a porcelain milk-ashing dish. Radioactivity was determined by means of a thin mica window Geiger-Müller tube.

As is shown in Table VII, the butyl alcohol extractability of the iodine in

rat plasma is quantitatively similar to that of human plasma. Almost all of the iodine passed into butyl alcohol, and about 84 per cent resisted further extraction with the 4 N NaOH-5 per cent Na_2CO_3 reagent. The results with the radioactive iodine were somewhat different. Only 72 per cent passed into the butyl alcohol, and 66 per cent remained in the butanol after the latter was subjected to extraction with alkali.

In seeking an explanation for the apparent discrepancy between the chemical and the radioactive data the following factors must be considered. (1) Not all of the iodine in the rat plasma had the same specific activity. If, for example, a small amount of diiodotyrosine-like iodine leaked into the plasma from the gland, this iodine would have a higher specific activity than the iodine of the thyroxine fraction (22). The injected iodide that was still present in the plasma would, of course, also have a much higher specific activity than the thyroxine iodine. (2) Inorganic iodide and diiodotyrosine, the compounds with the highest specific activity, are extractable from plasma with butyl alcohol to a lesser degree than thyroxine.

In the light of these considerations it would be expected that the chemical iodine in the plasma of the injected rats would be extracted with butyl alcohol to a greater extent than the radioactive iodine. Whether this alone accounts entirely for the discrepancy is difficult to say.

The possibility of some error (5 to 10 per cent) in the chemical method used here must also be considered in seeking to explain the discrepancy between the chemical and the radioactive data. Admittedly, the determination of 0.1 to 0.2 γ of iodine in the butyl alcohol extracts was not a simple matter, but it must be added that such analyses were always done in triplicate or quadruplicate. It seems unlikely to us that the average value of 80 per cent for the butyl alcohol-soluble, alkali-insoluble fraction of plasma iodine suffered from an error of more than 5 per cent.

Recrystallization of Thyroxine Carrier Added to Butanol Extracts—Butyl alcohol extracts, reextracted with the alkali reagent, were prepared from the plasma of rats injected 24 hours previously with 75 to 80 microcuries of I^{131}. 25 mg. of recrystallized thyroxine were added as carrier, and the butyl alcohol extract was concentrated to dryness on a boiling water bath under reduced pressure. The thyroxine in the residue was dissolved in hot 0.1 N K_2CO_3 and separated from a small amount of undissolved material by centrifugation. The thyroxine was then repeatedly recrystallized as follows: (1) The first crystallization was effected by the addition of glacial acetic acid to the hot K_2CO_3 solution of the residue. (2) The crystals obtained in this way were washed with water, dissolved in a minimum quantity of hot 0.1 N K_2CO_3, and then precipitated by cooling to 0°. (3) The crystals obtained in (2) were dissolved in an alkaline 70 per cent ethyl alcohol solution and precipitated by the addition of glacial acetic

acid. (4) The fourth recrystallization was made from a dilute NaOH solution by the addition of glacial acetic acid.

The specific activity of the iodine of each batch of crystals (counts per second per microgram of I^{127}) was determined. The results are recorded in Table VIII. The constancy in the values indicates that the I^{131} in the butyl alcohol extract of rat plasma is in the same form as the material which underwent recrystallization, namely thyroxine. Such an experiment is not absolutely conclusive, however, since a compound very similar to thyroxine in its structure might conceivably continue to precipitate with thyroxine.

Solvent Distribution Experiment—To provide further evidence as to whether the radioactive iodine in the butyl alcohol extract of plasma was

TABLE VIII

Specific Activity of Thyroxine Carrier after Successive Recrystallizations
See the text for details.

Experiment No.	Per cent of total I^{127} in BuOH fraction	Per cent of total I^{131} in BuOH fraction	Specific activity, counts per min. per γ I				
			Initial	After 1st recrystallization	After 2nd recrystallization	After 3rd recrystallization	After 4th recrystallization
1	84	66	2.64	2.58	2.58	2.47	2.53
2	84	66	2.57	2.25	2.20	2.28	2.28
3			2.90	2.69	2.81	2.74	2.70
4			2.89	2.69	2.71	2.66	2.63

thyroxine, its distribution between two immiscible solvents was compared with that of added thyroxine carrier. The carrier was added to the butyl alcohol extract containing the radioactive iodine and the mixture concentrated to dryness on a boiling water bath under reduced pressure. The residue was dissolved in 15 cc. of 0.1 N NaOH and shaken with an equal volume of either butyl alcohol or isoamyl alcohol. The distribution of chemical and radioactive iodine between the organic and the aqueous phases is recorded in Table IX. It was found that the distribution of the I^{131} always paralleled that of the added thyroxine.

This parallelism is all the more striking, inasmuch as the distribution ratios varied from experiment to experiment. The variation suggests that a variable breakdown or transformation of the thyroxine occurs during the process of concentrating the alkaline butyl alcohol extracts to dryness. Despite these variations, however, the distribution ratios for radioactive and chemical iodine in any given experiment were about equal. Such results would be expected only when the radioactive iodine was of the same molecular species as the added carrier.

Comparison of Behavior of Thyroxine and Thyroxine Peptide As Judged by Solvent Distribution Experiments—The evidence outlined above is best explained by the assumption that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein. The question arises, however, whether a thyroxine-containing peptide is ruled out by the evidence at hand. To provide information on this point a thyroxine peptide was prepared by the procedure of Harington and Salter (26). Desiccated thyroid powder obtained from the Viobin Corporation was first hydrolyzed with pepsin (Merck) and then with pancreatin (Merck). A product was finally isolated which closely resembled that obtained by Harington and Salter. It contained 49.4 per cent I, 3.4 per cent N, 1.3 per cent amino N, and gave a

TABLE IX

Distribution of Crystalline Thyroxine Carrier and Radioactive Plasma Iodine between Two Immiscible Solvents

See the text for discussion.

Experiment No.	Amount of thyroxine carrier added	Concentration of I in organic solvent / Concentration of I in aqueous solution	
		Chemical I	Radioactive I
	mg.		
1*	10	1.67	1.78
2*	10	1.73	1.73
3*	1.5	1.43	1.35
4*	0.9	0.81	0.84
5†	10	0.069	0.072

* Solvent pair, 0.1 N NaOH-*n*-butyl alcohol.
† Solvent pair, 0.1 N NaOH-isoamyl alcohol.

strong nitrous acid color test for thyroxine. But it also gave a positive ninhydrin test, which indicates the presence of some free amino acid. If this free amino acid is assumed to have a molecular weight of 120 (average for ordinary amino acids), then it represents an impurity of about 6 per cent. If it is assumed to be free thyroxine, however, it would have amounted to about 40 per cent contamination because of the large molecular weight of thyroxine (777). The small amount of material available did not permit us to carry out all the analyses necessary to establish the true identity of this product. But it is safe to assume that it was composed mainly of thyroxine peptides, the ratio of total N to amino N (2.6) leading to the conclusion that it was a mixture of di- and tripeptides.

A solvent distribution experiment was carried out with this preparation in the manner described above for thyroxine, except that the butyl alcohol extract, after the addition of the carrier, was not concentrated to

dryness but shaken directly with 0.1 N NaOH.   This avoided the possibility of chemical changes that might occur during the concentration.   The results for both peptide carrier and thyroxine carrier are presented in Table X.   It is evident that the radioactivity distributed itself very much like the thyroxine carrier but quite differently from the peptide carrier. *This demonstrates the sensitivity of this procedure in distinguishing between thyroxine and a closely related compound and lends further support to the view that the circulating thyroid hormone is actually thyroxine.*

It will be noted that the distribution ratios of thyroxine recorded in Table X are quite different from those in Table IX.   In the former are recorded the results of the experiments in which the butyl alcohol extracts containing the thyroxine were not concentrated to dryness; the values found for the ratio of the concentration of I in butyl alcohol to the concentration

TABLE X

Comparison of Crystalline Thyroxine and Thyroxine Peptide Preparation in Solvent Distribution Experiment

Solvent pair, 0.1 N NaOH-n-butyl alcohol.

Sample No.	Carrier added	Amount of carrier added	Concentration of I in organic solvent Concentration of I in aqueous solution	
			Chemical I	Radioactive I
		mg.		
1	Thyroxine	0.6	3.2	3.0
2	"	0.6	3.1	3.0
3	"	0.6	3.0	3.0
4	Peptide	1	1.5	3.1
5	"	1	1.5	3.2

of I in 0.1 N NaOH were much higher in these experiments than in those of Table IX.   As already pointed out, the lower values observed in the experiments of Table IX may be attributed to some transformation of thyroxine which occurs while the butyl alcohol extract is being concentrated to dryness and which renders the iodine less soluble in butyl alcohol.

Combination of Thyroxine with Plasma Proteins

The data so far presented lead to the conclusion that thyroxine in a loose combination with plasma protein is the circulating thyroid hormone. It seems of interest, therefore, to determine the particular fraction of plasma proteins with which thyroxine is combined.

Iodine Content of Plasma Protein Fractions—Salter and his coworkers (18, 27) have carried out iodine analyses on the various plasma protein fractions prepared by Cohn (28).   The two albumin fractions, Nos. V and

VI, were found to contain 75 per cent of the protein-bound iodine. The concentration of iodine appeared to be greatest, however, in the α-globulin fraction.

These results have been confirmed by us (Table XI). The following plasma fractions were analyzed: crystalline bovine albumin, human albumin (Fraction V), human α-globulin (Fraction IV-1), human β-globulin (Fraction IV-3,4), and human γ-globulin (Fraction II). The α-globulin fraction had the highest concentration of iodine (1.9×10^{-4} per cent), the γ-globulin the lowest ($<1 \times 10^{-5}$ per cent). The iodine content of the albumin fraction was, unfortunately, too small for accurate analysis, and the value recorded is probably too low. Nevertheless the albumin fraction is the largest carrier of iodine because it constitutes the largest fraction of

TABLE XI

Iodine Content of Plasma Protein Fractions

See the text for discussion.

Fraction No.	Principal components	Iodine per 100 gm. protein	Approximate protein per 100 cc. plasma	Estimated I per 100 cc. plasma
		γ	gm.	γ
IV-1	α-Globulin β-Globulin	189	0.5	1
IV-3,4	β-Globulin α-Globulin	56	0.8	0.5
II	γ-Globulin	<10	0.8	0
V	Albumin	35	4	1.5
Crystalline bovine albumin	"	30		
I	Fibrinogen	<10	0.4	0

the plasma proteins. The values given in Table XI should, however, be regarded as preliminary observations; more complete results must await further refinements in the iodine method as well as a more abundant supply of pure α- and β-globulins.

Thyroxine-Protein Combination—Many reports have dealt with the combination of various molecules with plasma proteins. Thus Roberts and Szego (29) reported that circulating estrogen is attached to plasma protein, probably the β-globulin fraction, whereas the combination of many organic anions with serum albumin has been studied by Klotz *et al.* (30), Teresi and Luck (31), and others. Davis (32) has discussed the physiological significance of the binding of molecules by plasma proteins.

In Table XII are presented our results on the binding of thyroxine by various plasma protein fractions. 100 mg. of each protein were dissolved or suspended in 10 cc. of a phosphate-saline (0.01 M phosphate-0.15 M

NaCl) buffer at pH 7.3. To 3 cc. of a protein solution in a small dialysis bag (Visking casing) was added 1 cc. of a standard thyroxine solution. Dialysis was carried out in a cold room (6–7°) against 100 cc. of phosphate buffer or distilled water. After 4 hours the external solution was replaced with a fresh 100 cc. portion and the dialysis continued for another 12 hours. The bag was rotated by motor during the dialysis. It is clear from the data in Table XII that albumin, α-globulin, and β-globulin are all capable

TABLE XII

Combination of Thyroxine with Plasma Proteins

Thyroxine was added to a 1 per cent protein solution in a phosphate-saline buffer at pH 7.3 and dialyzed. See the text for details.

Protein fraction	Thyroxine added	Dialyzed against	Per cent of added thyroxine remaining in dialysis bag
	mg.		
None ...	26.2	Distilled water	3.2
Albumin, Fraction V	21.4	" "	98
" " " .	21.4	Phosphate-saline buffer, pH 7.3	82
α-Globulin, Fraction IV-1	21.4	Distilled water	86
" " "	21.4	Phosphate-saline buffer, pH 7.3	81
β-Globulin, " IV-3,4	25.2	Distilled water	91
" " "	24.5	Phosphate-saline buffer, pH 7.3	70
γ-Globulin, " II	25.2	Distilled water	58
" " "	24.5	Phosphate-saline buffer, pH 7 3	22

of binding thyroxine to a high degree. γ-Globulin is much less potent as a thyroxine binder.

It may be concluded that plasma iodine is not confined to a single plasma protein fraction. Albumin, α-globulin, and, probably to a lesser extent, β-globulin share in binding the so called "protein-bound iodine" of plasma. γ-Globulin seems to play no rôle in this respect. It does seem, however, as pointed out by Salter (27), that the iodine in plasma is attached to the smaller protein molecules.

DISCUSSION

The concentration of "protein-bound" iodine in plasma is now widely used for the diagnosis of thyroid diseases (33–37).[1] This iodine is the frac-

[1] Chaney, A L , private communication (1944).

tion that is precipitated from plasma along with the proteins by such agents as zinc hydroxide, tungstic acid, or acetic acid plus heat, the inorganic iodine being freed from the protein precipitate by simple washing. Protein binding, however, is such a non-specific reaction that the finding of iodine in the plasma protein precipitate does not contribute much to an understanding of the chemical nature of this iodine.

Alcohol and acetone have been widely employed to fractionate the iodine of plasma ((9) p. 72, (38–40)). But such solvents do not achieve a clear cut separation of organic from inorganic iodine of plasma. Their inadequacy has been pointed out by Trevorrow (5), Salter ((9) p. 72), and others (23, 24, 38). Butyl alcohol is much more satisfactory for this purpose, since it has been successfully applied to the thyroid gland and since inorganic and non-thyroxine organic iodine can be reextracted from butyl alcohol by shaking this solvent with appropriate reagents.

The first to apply Leland and Foster's butyl alcohol fractionation procedure to blood iodine were Elmer et al. (17). They reported that after strong alkali hydrolysis (which destroyed part of the thyroxine present) 40 to 60 per cent of the organic iodine in the blood was thyroxine-like as judged by solubility properties. However, their values for normal human blood are high enough to cast suspicion upon their method for the determination of iodine.

Some time later, Trevorrow (5) also applied Leland and Foster's method to beef plasma and whole blood. She concluded that all of the iodine in plasma or whole blood could be directly extracted with butyl alcohol at room temperature and without previous hydrolysis. After reextraction of the butyl alcohol with 2 N NaOH, which served to remove inorganic and diiodotyrosine iodine, a good part of the iodine remained in the butyl alcohol. Her data did not permit quantitative conclusions regarding the percentage of total iodine in the thyroxine fraction.

Bassett, Coons, and Salter (18) also applied a butyl alcohol fractionation to plasma that had been subjected to pepsin hydrolysis. They reported that the thyroxine-like fraction in normal human plasma amounted to 73 per cent of the protein-bound iodine, but the range of values and number of patients studied were not given. Interestingly enough, approximately this same percentage was found in the thyroxine fraction in three cases of hyperthyroidism.

Somewhat different results were reported by Wilmanns in an extensive study of the iodine in normal, hyperthyroid, and hypothyroid blood (19). He treated whole blood with hot butyl alcohol and reported that only 65 per cent of the iodine could be removed from normal blood with this solvent, and of this only 28 per cent was not reextractable with 1 N NaOH. He concluded that there are at least two organic iodine fractions in whole

blood: (1) free thyroxine (average 28 per cent) and (2) a stable protein-bound iodine fraction (average 34 per cent).

The results obtained in the present investigation confirm Trevorrow's observation that almost all of the iodine in plasma can be extracted with butyl alcohol at room temperature. This finding leads us to conclude that the iodine of plasma is not stably bound to protein and that its state thus differs from that in which iodine exists in the thyroid gland. The iodine of the gland becomes butyl alcohol-soluble only after strong hydrolysis. Not only is most of the iodine in plasma extractable with butyl alcohol, but, as Table I shows, only 10 to 15 per cent of it can be removed from this solvent with the reagent 4 N NaOH-5 per cent Na_2CO_3, which extracts inorganic iodine and diiodotyrosine, but not thyroxine, from butyl alcohol. In seven human subjects and in one sample of pooled rat plasma 73 to 93 per cent of the total plasma iodine (average 81 per cent) remained in the butyl alcohol extract after it had been treated with the alkali mixture. When crystalline thyroxine was added to plasma in physiological amounts and the mixture subjected to the butyl alcohol extraction procedure, 77 to 78 per cent of the thyroxine appeared in the final butyl alcohol extract. Such findings demonstrate that a large fraction (at least 80 per cent) of plasma iodine behaves like thyroxine in its solubility properties. The additional findings that thyroxine added to plasma precipitates quantitatively with the proteins (Table IV) and does not dialyze (Table V) also lend support to the view that the iodine in plasma is mainly in the form of thyroxine loosely attached to protein.

Further evidence that thyroxine exists in plasma is provided by the experiments with radioactive iodine (Tables VIII to X). Crystalline thyroxine carrier when added to the butyl alcohol extract of the plasma of rats injected with I^{131} showed a constant specific activity upon repeated recrystallization. Even more convincing, perhaps, was the finding that the radioactive iodine in the butyl alcohol extract distributes itself between two immiscible solvents in almost exactly the same manner as does added thyroxine carrier (Table IX), but quite differently from a thyroxine peptide carrier (Table X).

The results obtained here agree quite well with those of Bassett, Coons, and Salter (18), although these workers hydrolyzed the plasma protein with pepsin before extraction with butyl alcohol. Apparently, preliminary hydrolysis of the proteins does not appreciably affect the free thyroxine content of plasma.

Although Trevorrow reported that practically all of the iodine in small samples of blood or plasma could be extracted with butyl alcohol, she found that in the case of beef plasma a variable but large portion (42 to 73 per cent) of the butyl alcohol-soluble iodine could be reextracted with 2

N NaOH. Most of the values, however, were based on experiments in which large volumes of plasma were used and extraction with butyl alcohol was admittedly incomplete. Nevertheless, in view of this discrepancy between our results and Trevorrow's, the possibility of species variation must be considered.

In a recent paper dealing with the effects of thyroxine, thyroglobulin, and thyrotropin on tissue respiration (41), Williams and Whittenberger were led to the conclusion that the active form of the thyroid hormone is probably a thyroxine peptide. Their main evidence is that thyroxine had no calorigenic effect on liver tissue slices, whereas thyroglobulin[2] did. They also reported that the serum of myxedematous patients given thyroxine intravenously gradually acquired the capacity to raise the QO_2 of guinea pig liver slices incubated therein, the maximum effect being reached in 6 hours. The latter experiment was taken as evidence that injected thyroxine must undergo some process of activation before it can increase oxygen consumption. Peptide formation was suggested as a possible mode of activation. Salter also appears to favor the peptide hypothesis (27).

The results obtained by us favor thyroxine rather than thyroxine peptides as the chemical form of plasma iodine. If any activation of thyroxine is required before it can act on tissues, it seems unlikely that peptide formation is involved. More likely the solubility of thyroxine and its rate of penetration to the site of reaction are important.

Harington has reported immunological experiments which support the view that thyroxine itself circulates in the plasma (14). He found that antisera against artificial thyroxine-protein complexes protected rats against the characteristic response to a dose of thyroxine. He has also reviewed the evidence *against* the view that thyroxine itself is the normal circulating hormone and no longer finds it convincing. Nevertheless the whole question of the relation between the biological activity of thyroid preparations and their thyroxine content deserves accurate reinvestigation with proper attention to the effects of route of administration, solubility, relative activities of D- and L-thyroxine, accurate chemical thyroxine determinations, and method of biological assay.

The finding that the form in which thyroxine exists in plasma differs from that in the gland raises the question by what mechanism the hormone is released into the circulation. De Robertis and Nowinski (43) have reported the presence of a proteolytic enzyme in the gland which hydrolyzes thyroglobulin into smaller fragments. The concentration of this enzyme supposedly is increased in hyperthyroidism. Confirmation of these interest-

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[2] The claim that the addition of thyroglobulin increases the respiration of liver slices has not been confirmed (42).

ing findings would be welcomed as a further step toward an understanding of the workings of the thyroid gland.

SUMMARY

The following evidence is presented in support of the view that the circulating thyroid hormone in the normal animal consists of thyroxine loosely attached to plasma protein.

1. The iodine of normal plasma is almost completely extractable with butyl alcohol at room temperature. Most of this iodine (73 to 93 per cent) remains in the butyl alcohol even after the latter is shaken with 4 N NaOH-5 per cent Na_2CO_3, a reagent which extracts diiodotyrosine and inorganic iodine, but not thyroxine, from butyl alcohol.

2. When crystalline thyroxine is added to plasma, it behaves like naturally occurring protein-bound iodine of plasma in the following respects: (a) Approximately 80 per cent of it remains in the butyl alcohol extract after treatment with the 4 N NaOH-5 per cent Na_2CO_3 reagent. (b) It precipitates quantitatively with plasma proteins when $Zn(OH)_2$ is used as the precipitating agent. (c) It does not dialyze.

3. Protein-bound iodine of rat plasma labeled with I^{131} follows thyroxine carrier quantitatively when the latter is repeatedly crystallized or when it is distributed between two immiscible solvents.

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THE ISOLATION AND IDENTIFICATION OF 2,2'-DITHIOLISO-BUTYRIC ACID FROM ASPARAGUS*

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A non-protein sulfhydryl substance occurs in edible asparagus. Fresh press-juice gives a test for both sulfhydryl and disulfide groups, so that an equilibrium apparently exists in the plant between the sulfhydryl and the disulfide (oxidized) form. However, when press-juice is exposed to atmospheric oxygen, the sulfhydryl form goes over completely to the oxidized form. Attempts to identify this substance as cystine or oxidized glutathione either by isolation or by polarographic analysis have been unsuccessful.[1]

It has now been found that the disulfide compound is acidic, thus permitting its isolation and purification by the extraction of concentrated acidified asparagus juice with butanol, removal of the substance from the butanol with sodium bicarbonate solution, subsequent acidification of the bicarbonate solution, and reextraction of the disulfide compound therefrom with butanol. A repetition of this distribution with several solvents gave a material which appeared to be pure. However, it was not found possible to crystallize the disulfide compound or its derivatives such as the methyl ester, since in the formation of the disulfide material from the sulfhydryl compound a relatively large polymer appeared to form. Even ethereal solutions of the disulfide compound became viscous on standing.

However, upon reduction with sodium in liquid ammonia, 2,2'-dithiolisobutyric acid (I) was isolated in pure crystalline form. The proof of the structure of this material was obtained from the accompanying series of reactions. Hydrogenolysis of 2,2'-dithiolisobutyric acid (I) with Raney's nickel (1) gave isobutyric acid (III) which was identified as the p-phenylphenacyl ester (2). Refluxing (I) with formaldehyde (3) gave a product whose analysis was in agreement with that of the previously undescribed 1,3-dithiane-5-carboxylic acid (II). The reaction of the sodium salt of the reduced product in liquid ammonia with methyliodide (4) gave dimethyl-2,2'-dithiolisobutyric acid (IV). This was shown by

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[1] Potter, E. F., and Johnson, C. M., unpublished results.

the identity of the product so formed with synthetic dimethyl-2,2'-dithiolisobutyric acid, which was prepared from 2,2'-diiodoisobutyric acid (V). The 2,2'-diiodoisobutyric acid was synthesized by the oxidation of glycerol dichlorohydrin (VI) to dichloroacetone, cyanhydrin synthesis on this acetone derivative, subsequent hydrolysis to 1-hydroxy-2,2'-dichloroisobutyric acid, and finally reduction with fuming hydriodic acid (5).
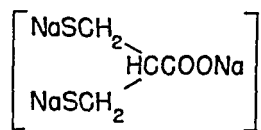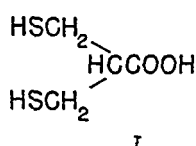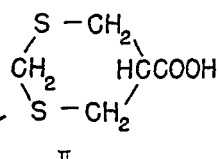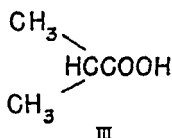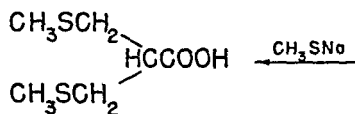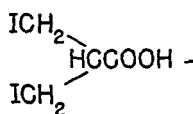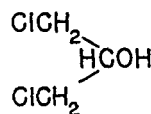
FIG. 1

EXPERIMENTAL

Isolation of Asparagus Disulfide Compound—9 gallons of commercial asparagus concentrate,[2] weighing 40 kilos, were diluted with an equal volume of water. This solution, which was at pH 4.5, was extracted with 7 gallons of butanol by agitation for 20 minutes.[3] After standing for 2 hours the liquid phases were separated by means of a large Sharples supercentrifuge. The disulfide compound was then extracted from the butanol with 3 gallons of dilute sodium bicarbonate solution. After acidification of the bicarbonate extract, the disulfide compound was removed from the aqueous solution with a total of 4 gallons of butanol. The butanol solution was

[2] The 66 brix concentrate (62.5 per cent solids) was prepared from asparagus butts by Libby, McNeill, and Libby of Sacramento, California.

[3] More of the disulfide compound could be extracted at a lower pH; however, relatively more impurities were likewise extracted.

concentrated to a volume of 270 ml. *in vacuo* with a bath at 35° and a dry ice-alcohol trap. A small amount of insoluble material was filtered off and discarded. To the filtrate, 1300 ml. of benzene were added and the precipitate formed was filtered off. The benzene solution was then shaken thrice with 330 ml. of 5 per cent sodium bicarbonate solution; the bicarbonate solution was washed with benzene and then acidified to pH 2 with 10 per cent sulfuric acid. Some sticky black insoluble material was immediately removed by centrifugation and filtration. The disulfide compound was extracted from the water solution with 2 liters of ether in three batches. The pale yellow ether solution was dried over anhydrous sodium sulfate and concentrated to a volume of 100 ml. This ethereal solution, which contained 0.317 gm. per ml., was used in the subsequent work. Hence from 40 kilos of concentrate (which corresponds to approximately 1400 kilos of asparagus butts) 31.7 gm. of the disulfide compound were isolated. Since at pH 4.5 the disulfide compound was approximately one-third titrated,[4] this yield would have been greater if it were feasible to extract the concentrate with butanol at a lower pH.[3]

Reduction of Disulfide Compound to 2,2'-Dithiolisobutyric Acid—To 175 ml. of liquid ammonia were added 13 ml. (4.1 gm.) of the ethereal solution of disulfide compound. Metallic sodium was added in small pieces until a relatively permanent blue color was obtained. As the reduction proceeded the material went into solution. The ammonia was allowed to evaporate spontaneously. The residue was taken up in 40 ml. of water and quickly acidified with concentrated hydrochloric acid to pH <2. The residue was allowed to stand overnight with 600 ml. of petroleum ether (b.p. 92–100°) and then reextracted with 200 ml. The petroleum ether was cooled to $-18°$ for several days. Beautiful colorless crystals of 2,2'-dithiolisobutyric acid separated. Upon recrystallization from 800 ml. of petroleum ether 1.97 gm. of 2,2'-dithiolisobutyric acid were obtained corresponding to a 48 per cent yield on a solid basis. The product melted at 61–62°.

Analysis—$C_4H_8O_2S_2$
Calculated.   C 31.56, H 5.30, S 42.12, mol. wt. 152
Found.   " 32.3, " 5.30, " 41.6, neutral equivalent 152, mol. wt.[5] 155, equivalent weight by —SH titration[6] 77

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[4] Unpublished results.

[5] The molecular weights were determined by the Clark modification (6) of the Signer isothermal distillation method.

[6] The —SH groups were titrated at pH 5.3 with *p*-chloromercuribenzoate with nitroprusside as an outside indicator according to the method of Hellerman *et al* (7). The *p*-chloromercuribenzoate was standardized with a specially purified sample of cysteine hydrochloride. The value found agreed to within less than 2 per cent of the amount of *p*-chloromercuribenzoate weighed out.

Reaction of 2,2′-Dithiolisobutyric Acid with Formaldehyde—The conditions used for this reaction were essentially those which Armstrong and du Vigneaud used for the formation of djenkolic acid from cysteine (3). To a solution of 0.25 gm. (1.6 mM) of 2,2′-dithiolisobutyric acid in 50 ml. of 0.1 N HCl was added 0.14 ml. (1.9 mM) of formalin, and the reaction mixture was refluxed for 2 hours (the reaction proceeded very slowly at 70°). The reaction mixture was evaporated to dryness *in vacuo*, whereupon a crystalline residue was obtained. The crystals were recrystallized from 12 ml. of hot water, yielding 0.19 gm. of needles melting at 143–146°. This yield corresponds to 70 per cent of the theoretical for the conversion to 1,3-dithiane-5-carboxylic acid. Upon recrystallization from 10 ml. of hot water the product melted at 146–148°. The analyses were in agreement with the composition of 1,3-dithiane-5-carboxylic acid.

Analysis—$C_5H_8O_2S_2$
        Calculated. C 36.56, H 4.91, S 39.04, mol. wt. 164
        Found.   " 36.7, " 5.04, " 39.8, neutral equivalent 161

This material no longer gave a nitroprusside test upon treatment with potassium cyanide. On an equivalence basis, it gave 65 per cent of the color that methionine gives in the Axelrod modification (8) of the McCarthy-Sullivan method for methionine (9). Axelrod[7] has shown that all the substances tested which contain a sulfur-methylene group gave this reaction.

Hydrogenolysis of 2,2′-Dithiolisobutyric Acid to Isobutyric Acid—The procedure used for the hydrogenolysis of 2,2′-dithiolisobutyric acid was the same that Mozingo *et al.* (1) employed for the hydrogenolysis of several sulfur compounds. To a suspension of 10 gm. of Raney's nickel (10) in 125 ml. of ethanol, 0.5 gm. (3.3 mM) of 2,2′-dithiolisobutyric acid and 75 ml. of water were added and the reaction mixture was refluxed for 2 hours. The nickel was filtered off and washed with 500 ml. of 0.2 N and 100 ml. of 1 N NaOH. The filtrate and washings were combined and the alcohol was distilled off of the alkaline solution. The aqueous solution was acidified with sulfuric acid and the isobutyric acid was steam-distilled. After neutralization of the distillate it was concentrated *in vacuo* to approximately 5 ml. and then made slightly acidic with 1 N hydrochloric acid. To this solution were added 0.907 gm. (3.3 mM) of *p*-phenylphenacyl bromide and 10 ml. of ethanol, and the reaction mixture was then refluxed for 1 hour (2), whereupon an oil separated. Sufficient 60 per cent ethanol was added to dissolve the oil when hot. On cooling overnight at 5°, 0.60 gm. of crystalline *p*-phenylphenacyl ester of isobutyric acid was obtained (65 per cent of theoretical), melting at 70–80°. After two recrystallizations from 60 per cent ethanol the melting point was constant at 88–90°.

[7] Axelrod, B., unpublished results.

The melting point of a mixture with an authentic sample of the ester was the same.

Analysis—$C_{18}H_{18}O_3$.   Calculated.   C 76.57, H 6.43
                              Found.   " 76.3, " 6.45

Preparation of p-Phenylphenacyl Ester of Isobutyric Acid—Since this ester has not previously been described, it was necessary to prepare it for identification purposes. For this purpose the method of Drake and Bronitsky (2) was employed with 0.01 mole of isobutyric acid and an equivalent amount of p-phenylphenacyl bromide. The procedure was the same as above. After two crystallizations, the melting point was constant at 88–90°.

Analysis—$C_{18}H_{18}O_3$.   Calculated.   C 76.57, H 6.43
                              Found.   " 76.4, " 6.26

Formation of Dimethyl-2,2'-Dithiolisobutyric Acid from Disulfide Compound—A metallic sodium reduction of 15 ml. of the ethereal solution of the disulfide compound was carried out as above. After the reduction was complete, 15 ml. of methyl iodide were slowly added to the liquid ammonia solution (4). The ammonia was allowed to evaporate spontaneously. The residue was dissolved in water and acidified with dilute hydrochloric acid to pH 2.0 and then evaporated *in vacuo* to dryness. The residue was extracted with 400 ml. of benzene, which dissolved the dimethyl-2,2'-dithiolisobutyric acid completely. Upon removing the benzene, 3.14 gm. of an oily product were obtained, corresponding to a yield of 60 per cent of theoretical. The oil was dissolved in 200 ml. of petroleum ether (b.p. 92–100°) at 25° and cooled to −18°, whereupon the dimethyl-2,2'-dithiolisobutyric acid crystallized out in elongated rectangles. After two more crystallizations the melting point was constant at 23.5–25°. The melting point of a mixture with synthetic dimethyl-2,2'-dithiolisobutyric acid was identical.

Analysis—$C_6H_{12}O_2S_2$
Calculated.   C 39.97, H 6.71, S 35.57, CH_3 16.67, mol. wt. 180
Found.   " 40.0, " 6.83, " 34.9[8] CH_3[9] 14.3, neutral equivalent 179,
              mol. wt.[5] 183

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[8] These sulfur determinations were made by the Potter and Jones modification (11) of the Pollock and Partansky permanganate method (12), since this method is better suited for the determination of sulfur in substances which are oils at room temperature than is the Parr bomb peroxide method.

[9] The methyl determinations were carried out according to the Baernstein method for methionine (13) which gives results 6 per cent low (14). Substances other than

The dimethyl-2,2'-dithiolisobutyric acid gave 1.44 times as much color as did an equivalent amount of methionine in the modified (8) McCarthy-Sullivan method.

Synthesis of Dimethyl-2,2'-Dithiolisobutyric Acid—2,2'-Diiodoisobutyric acid was prepared from glycerol dichlorohydrin according to the directions of Glattfeld and Schneider (5), except that the cyanhydrin synthesis was carried out with the sodium bisulfide addition product of dichloroacetone and potassium cyanide (15) instead of dichloroacetone and liquid hydrogen cyanide.

To 125 ml. of a cold solution of sodium ethylate, prepared by reaction of 0.5 gm. (0.022 mole) of metallic sodium with ethanol, were added 9.5 gm. (0.195 mole) of methyl mercaptan. After a few minutes 2 gm. (0.006 mole) of 2,2'-diiodoisobutyric acid were added and the reaction mixture was allowed to warm to room temperature for 1 hour and then was refluxed for 6 hours. After acidification the reaction mixture was evaporated to dryness *in vacuo*. The residue was extracted with 100 ml. of warm petroleum ether (b.p. 92–100°) and the extract filtered. On cooling to −18° overnight only an oil separated. This oil was removed and the mother liquor was evaporated to a volume of 25 ml. On cooling again to −18°, 0.311 gm. of crystals melting at 23–24.5° was obtained. The yield of crystalline dimethyl-2,2'-dithiolisobutyric acid thus was 30 per cent of theoretical. After two recrystallizations, which were necessary for removal of the yellow color, the melting point was 23.5–25°.

Analysis—$C_6H_{12}O_2S_2$
    Calculated.   C 39.97, H 6.71, S 35.57, CH_3 16.67, mol. wt. 180
    Found.   " 39.8, " 6.76, " 35.0,[8] CH_3[9] 14.3, neutral equivalent 179

The synthetic dimethyl-2,2'-dithiolisobutyric acid under investigation gave the same amount of color in the modified McCarthy-Sullivan methionine method as did the substance prepared from the natural product.

DISCUSSION

2,2'-Dithiolisobutyric acid appears to be the first dithiol substance reported from a natural source. Because of its apparent similarity to British anti-lewisite (2,3-dimercaptopropanol) its rôle in the metabolism of asparagus may well be as an enzyme regulator in that it might control heavy metal inhibition. Or, since freshly pressed asparagus juice gives a test for both free sulfhydryl and disulfide groups, it may function as a

methionine may well give still lower results, particularly those containing two—SCH_3 groups. Hence these results cannot be considered as quantitative but only as establishing that two —SCH_3 groups occur in this compound.

hydrogen transfer substance similar to glutathione. The effect of this substance on respiration and on isolated enzymes will be investigated.

Other sulfur compounds which might be called unusual have been isolated from plant sources. Haagen-Smit et al. (16) have isolated methyl-β-methyl thiolpropionate from the volatile constituents of pineapple. Kröner and Wegner (17) have obtained concentrates of sulfur compounds from potatoes which are responsible for at least part of the odor and taste. It is noteworthy that one of the fractions of such compounds gave a direct reaction with silver foil. Hence there exists a variety of sulfur-containing substances in plants which undoubtedly contribute to the taste. Alterations in these substances during processing or storage of fruits and vegetables would most likely lead to off flavor formation.

Dithiolisobutyric acid, which for a mercaptan has relatively slight odor, apparently is not the precursor of the characteristic odor of the urine resulting after the ingestion of asparagus, since no odor resulted when two individuals took 10 mg. each of dithiolisobutyric acid orally.

SUMMARY

2,2'-Dithiolisobutyric acid has been isolated from asparagus by reduction of the oxidized form with metallic sodium. Proof of the structure of this substance was obtained from the following reactions. Hydrogenolysis of the 2,2'-dithiolisobutyric acid with Raney's nickel gave isobutyric acid (identified as the p-phenylphenacyl ester). Reaction with formaldehyde led to the formation of 1,3-dithiane-5-carboxylic acid. The sodium salt of the reduced disulfide substance obtained from asparagus, when treated in liquid ammonia with methyl iodide, gave dimethyl-2,2'-dithiolisobutyric acid. The acid so obtained was identical with dimethyl-2,2'-dithiolisobutyric acid prepared synthetically from glycerol dichlorohydrin.

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DIPHOSPHOPYRIDINE NUCLEOTIDE PYROPHOSPHATASE

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Enzymatic splitting of diphosphopyridine nucleotide (DPN)[1] by micro-organisms, plants, and animal tissues has been known for many years, but the precise nature of this reaction is not well understood (1). Ohlmeyer (2) first observed that adenylic acid (5-phosphoadenosine) appeared when DPN was incubated with yeast maceration juice. Later Heiwinkel (3) identified adenylic acid as a product of the action of sweet almond press juice on DPN. Although free nicotinamide did not appear, the presumed nicotinamide mononucleotide could not be isolated. Das and von Euler (4, 5) found inorganic orthophosphate as a product of DPN destruction in animal tissues, but there was no indication as to the mechanism of this reaction. Handler and Klein (6) recovered free nicotinamide quantitatively as a product of DPN destruction by brain, liver, kidney, and muscle preparations from rabbits, rats, and dogs, and concluded that the nicotinamide-ribose linkage was the primary and principal site of cleavage in these tissues.

In the course of studies of oxidative phosphorylation, we observed that washed particles of rabbit kidney converted added DPN to adenosine triphosphate (ATP). In the absence of an oxidizable substrate to provide an energy source for phosphorylation, adenylic acid was formed in place of ATP. The other product of DPN splitting was tentatively identified, after partial purification, as nicotinamide ribose phosphate. These and related observations make it probable that in such rabbit kidney preparations cleavage of the pyrophosphate bond is the predominant mechanism of DPN splitting. Almost all of the DPN-splitting activity of rabbit brain is due to nucleosidase activity.

Methods

Washed Particles of Kidney Cortex—Kidneys of a young rabbit were chilled on ice. All subsequent operations were performed at 2°. The cortex was homogenized in 15 ml. of 0.1 M potassium phosphate buffer of

[1] Nicotinamide-ribose-phosphate-phosphate-ribose-adenine.

pH 7.7 and centrifuged at 1500 R.P.M. for 5 minutes to remove coarse particles. The supernatant fluid was centrifuged at 10,000 R.P.M. for 30 minutes, and the particles obtained were washed three times with 1 per cent KCl and centrifuged each time at 10,000 R.P.M. for 15 minutes. They were suspended in 5 ml. of 0.1 M potassium phosphate buffer of pH 7.7, or in 5 ml. of 0.1 M NaHCO₃ (saturated at 22° with 5 per cent CO_2 + 95 per cent N_2). 1 ml. of $MgCl_2$ (0.03 M), 1 ml. of NaF (0.1 M), and water to 10 ml. were added. This kidney particle suspension contained 30 to 40 mg. of protein per ml. (Washed particles from rabbit brain were similarly prepared.) Unwashed homogenates in 0.1 M potassium phosphate buffer of pH 7.7 were used in some experiments.

Diphosphopyridine Nucleotide—DPN of 0.60 to 0.72 purity was prepared according to Williamson and Green (7) with minor modifications. $DPNH_2$ of purity 0.50 was prepared by Ohlmeyer's method (8).

Determinations—Incubations were carried out at 37°, with shaking, in air or O_2. Usually the reaction was stopped by trichloroacetic acid; for $DPNH_2$ determinations, however, the reaction was stopped by heating for 3 minutes at 100°. DPN was determined by the method of Cori, Slein, and Cori (9) at 340 mμ in the Beckman spectrophotometer. As an occasional check that the optical density increments were due only to $DPNH_2$, pyruvate and lactic dehydrogenase[2] were added when the reaction was complete. The densities invariably returned to the initial values. $DPNH_2$ was determined in the lactic dehydrogenase system as described by Kubowitz and Ott (10).

Compounds containing the nicotinamide-ribose (N-R) moiety were determined by the fluorometric method of Huff and Perlzweig (11) in the Coleman photofluorometer, with DPN as a standard. Under the conditions of the method, the formation of a highly fluorescent condensation product with acetone is specific for pentavalent N^1-substituted nicotinamide derivatives. In expressing results, it has been assumed arbitrarily that N-R-containing compounds arising from DPN splitting have a molar fluorescence equal to that of DPN.[3]

Phosphate was determined by the method of Fiske and Subbarow (12). Acid-labile phosphate was taken to be the phosphate liberated after 20 minutes hydrolysis in 1 N H_2SO_4 at 100°. Acid-stable phosphate was determined as the additional phosphate liberated after ashing with an H_2SO_4-HNO_3 mixture. The procedure of Cohn and Kolthoff (13) was adapted for the estimation of inorganic pyrophosphate. Pentose was determined by

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[2] Purified from rabbit muscle extract.

[3] DPN gave 1.4 to 1.9 times as much fluorescence, expressed on a molar basis, as N^1-methylnicotinamide chloride. The latter compound was kindly supplied by Dr. V. A. Najjar.

the method of Mejbaum (14) and adenylic acid by the method of Kalckar (15).

Results

Splitting of DPN by Rabbit Kidney and Recovery of Adenylic Acid— Preparations of washed particles of rabbit kidney invariably split added DPN. This destruction was generally associated with little or no decrease in concentration of the N-R moiety. Of twenty preparations incubated for a time interval sufficient to destroy 50 per cent or more of the DPN, the N-R value was decreased by more than 20 per cent in only four. The N-R value was increased by more than 10 per cent in two, and remained nearly constant (±10 per cent) in ten. These findings will be considered again later in this report.

The loss of DPN was accompanied by the appearance of nearly equivalent amounts of adenylic acid. Thus, after 15 and 30 minutes incubation, 1.75 and 2.44 micromoles of DPN were lost and 1.36 and 2.36 micromoles of adenylic acid, respectively, were recovered. Recoveries of adenylic acid after longer incubation periods were less complete, owing to the splitting of adenylic acid by nucleotidase.

The DPN-splitting activity of kidney particles was roughly proportional to their concentration. The activity was destroyed by heating at 100° for 2 minutes and was not significantly altered by omission of $MgCl_2$, NaF, or phosphate.

*Phosphate Balance and Separation of Reaction Products of DPN Splitting in-Non-Respiring System—*Without added substrate, there was practically no oxygen uptake by kidney particles, and the values for inorganic ortho-, acid-labile, and total organic phosphate were essentially unchanged during the splitting of DPN.[4]

Fractionation of the reaction products with mercury and lead resulted in partial separation of the three major components, adenylic acid, nicotinamide mononucleotide, and unsplit DPN (Table I). The incubation mixture was treated essentially as described below under "Purification of nicotinamide mononucleotide" through the mercury precipitation step. The insoluble mercury salts were freed of mercury and then fractionated with lead at pH 6.4. Adenylic acid was recovered as the predominant component of the insoluble lead salts, while DPN (unsplit) was recovered as a soluble lead salt. The soluble mercury fraction contained only small

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[4] While these results indicate that no significant amount of phosphate was liberated or esterified, small changes in phosphate concentration may have been obscured, since these experiments were carried out in 0.05 M phosphate buffer. In experiments with CO_2-bicarbonate buffer, up to 28 per cent of the phosphate content of the split DPN was liberated as inorganic orthophosphate. This indicates that some nucleotidase was present in these kidney preparations.

amounts of adenylic acid and DPN and consisted mainly of an N-R-containing compound. After refractionation with mercury, the latter fraction was found to contain phosphate, pentose, and N-R in roughly equivalent concentrations.

Stimulation of Respiration of Kidney Particles by DPN—The oxygen consumption and esterification of inorganic orthophosphate by rabbit kidney particles were markedly stimulated by DPN (Fig. 1, Table II). The rate of oxygen consumption in the presence of DPN showed no significant lag phase and remained constant throughout the period of observation. The stimulation of oxygen uptake by adenylic acid was somewhat less than with DPN and was not maintained so well. That the lesser effectiveness of adenylic acid was not due to a concomitant inhibitory effect is indicated

TABLE I

Separation of Reaction Products of DPN Splitting in Non-Respiring System

The incubation mixture contained 20.0 ml. of kidney particles suspended in 0.05 M phosphate buffer and 5.0 ml. of neutralized DPN solution. The values are expressed in micromoles.

	Adenylic acid	N-R*	DPN
Incubation mixture at 0 min.†	0	211	211
" " " 120 min.†	142	211	84
Mercury salt, soluble	9	95	6
" " insoluble; lead salt, insoluble	109	9	3
" " " " " soluble	4	79	70

* Nicotinamide-ribose moiety.
† Trichloroacetic acid filtrate.

by the rate of oxygen consumption with DPN and adenylic acid together, essentially the same as that for DPN alone and well maintained.

With graded amounts of DPN (Table II), the oxygen consumption was increased to a maximum value. A limit of phosphate esterification was not reached in these experiments and all the phosphate taken up was easily hydrolyzable in acid. Fractionation of the reaction products with barium will be considered below.

Phosphate Balance and Separation of Reaction Products of DPN Splitting in Respiring System—The addition of an oxidizable substrate, such as glutamate, to kidney particles resulted in active oxygen consumption and phosphate esterification. In the experiment summarized in Table III, approximately half the orthophosphate disappeared and could be accounted for as an acid-labile ester. Fractionation of the esterified phosphate indicated the presence of two or possibly three components. The neutralized trichloroacetic acid filtrate of the reaction mixture was treated with an

excess of barium acetate and the pH was adjusted to 6.9. The precipitate was dissolved in acid, the barium removed with H_2SO_4, and the pH buffered at 3.6. Addition of cadmium acetate, according to Cohn and Kolthoff (13), yielded a crystalline precipitate with only slight absorption at 260 mμ and can be presumed to be inorganic pyrophosphate (16). ATP is almost completely soluble as a cadmium salt at pH 3.6. Analysis of the phosphate ester not precipitated by cadmium indicated a composition con-
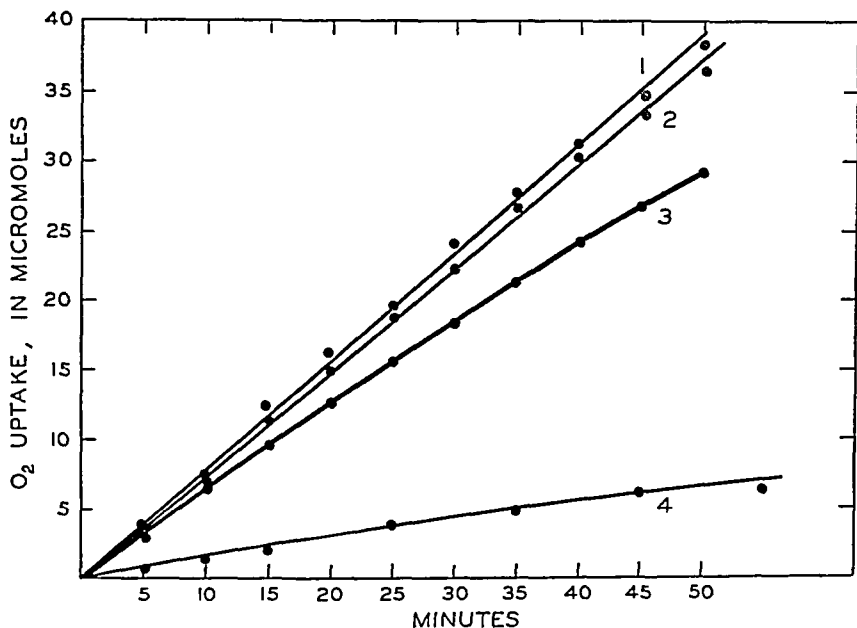
FIG. 1. Stimulation of respiration of kidney particles by DPN and adenylic acid. Curve 1, 2.5 micromoles of DPN; Curve 2, 2.5 micromoles of DPN + 2.5 micromoles of adenylic acid; Curve 3, 2.5 micromoles of adenylic acid; Curve 4, no additions. Each vessel contained 1.0 ml. of kidney particles, 130 micromoles of glutamate, the additions noted above, and water to make a final volume of 1.75 ml. Incubated in O_2 at 37°.

sistent with that of ATP. Analysis of the barium precipitate obtained by raising the pH to 8.4 also indicated ATP as the predominant organic phosphate ester. The presence of ATP in both fractions was checked qualitatively by the phosphorylation of glucose in the hexokinase reaction. The barium salts soluble at pH 8.4 contained almost all of the N-R and a small amount of acid-labile phosphate ester. The over-all recoveries of phosphate, pentose, and N-R were in fair agreement with the starting concentrations in the form of DPN.

The results of Table II are consistent with those of Table III, except that

the recovery of ATP was incomplete because the barium precipitate formed between pH 7 and 8.5 was not analyzed. Of some interest is the relative constancy of the acid-labile phosphate fraction precipitated by barium at pH 7, in which inorganic pyrophosphate is a prominent component (Table II). Possibly significant with respect to the question of whether nicotinamide mononucleotide is phosphorylated are the amounts of acid-labile

TABLE II

Effect of DPN on Oxygen Consumption and Phosphate Esterification

Each vessel contained 1.0 ml. of kidney particles in phosphate buffer, 130 micromoles of glutamate, and 4.2 micromoles of DPN at pH 7.7 in a volume of 1.3 ml. Incubation 60 minutes in O_2 at 37°. Corrections were made for the time elapsed in equilibration and fixation. Each sample was fixed with trichloroacetic acid and the filtrate fractionated with barium. Analyses were performed on the barium salts insoluble at pH 7 and on the barium salts soluble at pH 8.5. A barium precipitate which formed between pH 7 and 8.5 was not analyzed. The values are expressed in micromoles.

DPN added "　　remaining	4.2* 4 2	0 0	0.8 0	1.6 0	2.5 0	3 3 0	4.2 0	5.0 0	6.6 0.1	8 4 0.5
Oxygen uptake		6.4	22.5	26.5	29.5	33.1	33.6	34.5	37.1	37.1
Phosphate uptake	0	2.4	8.2	10.8	12.4	15.8	17.4	19.6	21.4	22.9
Acid-labile phosphate	1.3	3.3	8.2	10.8	12.2	15.8	17.4	18.8	21.6	23.6

Barium salt insoluble at pH 7

Acid-labile phosphate	0.4		5.9	6.3	7.9	8.3	8.4	8.5	7.9	8.3
Acid-stable　　"	0.3		0.3	0.4	0.7	0.9	1.2	0.5	1.1	1.6
N-R†	0	0	0	0	0	0	0	0	0	0

Barium salt soluble at pH 8.5

Acid-labile phosphate			0.1	2.2	3.0	2.9	3.1	3.7	4.7	5.8
Acid-stable　　"			2.6	3.2	4.3	4.9	7.5	8.2	8.5	13.3
N-R†	3.0	0	0.7	1.6	2.6	3.8	4.8	5.4	7.6	9.0

* The whole vertical column represents an unincubated control, fixed at zero time.
† Nicotinamide-ribose moiety.

phosphate in the barium salts soluble at pH 8.5, in which the N-R-containing compound is found (Tables II and III).

Purification of Nicotinamide Mononucleotide (NMN)—The procedure for separation of NMN from the other products of DPN splitting involved (1) deproteinization with trichloroacetic acid, (2) removal of barium salts insoluble at pH 8.4 (ATP, adenosine diphosphate, inorganic phosphate), (3) precipitation of the barium salt of NMN with acetone, (4) removal of compounds insoluble as mercury salts or complexes at pH 4 (DPN, adenylic acid), (5) adsorption of NMN on norit and elution with isoamyl alcohol.

Kidney particles in phosphate buffer (20 ml.) were incubated with DPN (400 micromoles) for 120 minutes at 37°. 25 ml. of cold 10 per cent trichloroacetic acid was added, and the resultant precipitate washed with cold 10 per cent trichloroacetic acid. The combined supernatant fluid and washings were neutralized, saturated barium acetate was added in excess, and the pH adjusted with NaOH to 8.4. The precipitate was washed with water and discarded. On addition of 500 ml. of cold acetone to the combined supernatant fluid and washings (87 ml.), a white flocculent precipitate formed, which turned to a yellow oil. This was centrifuged,

TABLE III

Phosphate Balance and Separation of Reaction Products of DPN Splitting in Respiring System

The incubation mixture contained 15 ml. of kidney particles in phosphate buffer, 1500 micromoles of glutamate, and DPN in a final volume of 18 ml. The mixture was shaken in oxygen. The values are expressed in micromoles.

	Phosphate			Ade-nine*	Pen-tose	N-R	DPN
	Ortho-	Acid-labile	Acid-stable				
Incubation mixture, 0 min.†	539	0	170‡	85‡	170‡	85	85
" " 60 " † ..	293	249					3
Barium salt, insoluble at pH 6.9							
Total insoluble barium salt	14	112		30		0	
Cadmium salt, insoluble		50		4			
" " soluble		71	31	25	28		
Barium salt, insoluble at pH 6.9–8.4	236	105	53	45	57	1	
" " soluble at pH 8.4	26	10	97		83	85	3

* Determined by measuring the absorption at 260 mμ with a coefficient of 1.6 $\times$ 10^7 sq. cm. $\times$ mole^{-1}.

† Trichloroacetic acid filtrate.

‡ Calculated from DPN determination; all other values are determined.

washed with acetone, dried *in vacuo*, and dissolved in dilute HCl. Approximately 80 per cent of the N-R originally present in the trichloroacetic acid extract was recovered.

Barium was removed with H_2SO_4 and the precipitate washed with water. Mercuric acetate was added until precipitation was complete (pH 3.80); analysis of the precipitate showed that the N-R lost in this step was almost entirely in the form of unsplit DPN. The supernatant fluid plus washings was freed of mercury with H_2S and shaken for 30 minutes with 2 gm. of norit (pH 2.0). Less than 1 per cent of the NMN was unadsorbed. The adsorbed NMN was eluted by shaking four times for 30 minutes with 10 ml. of 10 per cent isoamyl alcohol. The combined eluates, which contained

75 per cent of the N-R present before adsorption, were concentrated under reduced pressure to 8 ml.

Analysis of this purified sample gave the following values in micromoles per ml.: N-R 12.6, pentose 12.4, total organic phosphate 14.5, acid-labile phosphate 0.8, orthophosphate 0.0, and DPN 0.04. The ultraviolet absorption spectrum revealed a peak at 265 mμ. With the molecular extinction coefficient for the nicotinamide nucleoside of Schlenk (17), a value of 15.4 micromoles per ml. was obtained. Reduction of the purified NMN with hydrosulfite resulted in a marked but unstable increase in absorption between 280 and 380 mμ, with a maximum at about 330 mμ.

TABLE IV

Relative Rates of Splitting of DPN and DPNH$_2$

The incubation mixtures contained 0.20 ml. of kidney particles in phosphate buffer, 0.75 ml. of water, and 0.05 ml. of either DPN or DPNH$_2$. The initial values for DPN were 2.71, 2.54, and 2.54 micromoles and for DPNH$_2$ 3.22, 2.63, and 2.73 micromoles for Experiments 1, 2, and 3, respectively. The vessels were gassed with nitrogen passed over heated copper. The vessel contents to be analyzed for DPN were fixed by tipping in 0.5 ml. of 20 per cent trichloroacetic acid from a side arm; those to be analyzed for DPNH$_2$ were fixed by plunging the vessel, still attached to the manometer, into boiling water. The values are expressed in micromoles.

Experiment No.	Incubation time	Amount split	
		DPN	DPNH$_2$
	min.		
1	15	0.83	1.25
2	15	0.17	0.65
3	15	0.47	1.19
	30	0.85	1.48

Relative Rates of Splitting of DPN and DPNH$_2$—It was observed that the rate of DPN disappearance was more rapid (1.4 to 1.8 times) in an actively respiring system (*i.e.* glutamate present) than in one to which no oxidizable substrate had been added. That this disappearance of DPN was a splitting and not an accumulation of the reduced form was indicated by the absence of DPNH$_2$ at the end of the incubation period.

It was considered that the accelerated disappearance of DPN in respiring systems might be due to its conversion to DPNH$_2$ and to a more rapid splitting of DPNH$_2$. Das and von Euler (4) have reported that the enzymatic release of orthophosphate was faster from DPNH$_2$ than from DPN. The data in Table IV, obtained in the absence of oxidizable substrate, show that DPNH$_2$ is destroyed more rapidly than DPN. Anaerobiosis was complete enough to prevent any significant reoxidation of DPNH$_2$. These data thus provide a possible explanation for the accelerated splitting

of DPN in systems in which DPNH$_2$ is continuously being formed by substrate oxidation.

DPN Splitting by Rabbit Brain—In agreement with Handler and Klein (6), we have found splitting of DPN by brain more rapid than by kidney. While brain homogenate (0.5 ml. in an incubation mixture of 0.8 ml.) destroyed all but 0.8 of 8.9 micromoles of DPN after 30 minutes incubation, a similar concentration of kidney homogenate destroyed only 4.4 of 8.4 micromoles after 60 minutes incubation. Significant for the mechanism of DPN splitting by brain was the parallel disappearance of DPN and

TABLE V

DPN Splitting by Rabbit Brain

The incubation mixtures were as follows Experiment 1, 0 5 ml of brain particles (in phosphate buffer), 0.15 ml. of neutralized DPN solution, 0 20 ml of water, Experiment 2, 0.5 ml. of brain homogenate, 0 15 ml. of DPN, 0 10 ml of water, Experiment 3, 1.0 ml. of brain particles (in bicarbonate buffer), 0 30 ml of DPN, 0 20 ml of water; Experiment 4, 10 0 ml of brain particles (the same preparation as in Experiment 3), 0.30 ml. of crystalline muscle adenylic acid or purified nicotinamide mononucleotide, 0.20 ml. of water. The values are expressed in micromoles

Experiment No	Substance estimated	Values after incubation for			
		0 min	15 min	30 min	60 min
1	DPN	8 1	4 8	2 8	0 4
	N-R	8 1	4 9	2 6	0 5
2	DPN	8 9		0 3	
	N-R	8 9		0 3	
3	DPN	18 3	10 7	5 7	2 1
	N-R	18 3	12 2	7 4	2 4
4	NMN	12 3	3 0	1 1	0 7
	Adenylic acid	9.9	8 2	7 0	4 7

N-R (Table V). The rate of splitting of a purified preparation of NMN was even more rapid than that of DPN.

These preparations of brain particles also destroyed adenylic acid but at a relatively slow rate. Despite this slow rate of adenylic acid breakdown and the rapid splitting of DPN, adenylic acid could not be detected. In confirmation of Handler and Klein (6), no inorganic orthophosphate was detected during the splitting of DPN (nor was any inorganic orthophosphate found as a result of NMN splitting). Experiments with added adenylic acid revealed that adenylic acid disappearance agreed well, within 10 per cent, with release of inorganic orthophosphate.

Effects of Nicotinamide on DPN Splitting by Brain and Kidney—It has been reported (6, 18) that the inhibition by nicotinamide of DPN splitting by several tissues, including brain and kidney, is almost complete.

While these findings have been confirmed for brain, there was an almost complete lack of inhibitory effect by nicotinamide on DPN splitting by kidney particles (Table VI). The N-R values decreased at rates identical with DPN disappearance in experiments with brain, but, as stated in an

TABLE VI

Effects of Nicotinamide on DPN Splitting by Brain and Kidney

The incubation mixtures were as follows: Experiment 1, 0.30 ml. of brain (or kidney) particles in phosphate buffer ($MgCl_2$ and NaF omitted), 0.15 ml. of DPN, nicotinamide in the final concentrations shown, and water to a final volume of 0.55 ml. Experiment 2, 0.5 ml. of brain (or kidney) homogenate, 0.15 ml. of DPN, 0.08 ml. of 2 M nicotinamide, 0.07 ml. of water. Water replaced nicotinamide when the latter was omitted. The values are expressed in micromoles.

Tissue	Substance estimated	Experiment 1. Varying nicotinamide, 30 min. incubation					
		Nicotinamide					
		0.18 M*	0.18 M	0.09 M	0.045 M	0.018 M	0.000 M
Brain	DPN	7.7	8.2	7.7	7.7	6.2	3.5
	N-R	7.7	7.7	7.6	7.1	6.2	3.2
Kidney	DPN	7.4	5.4	5.6	5.6	5.9	5.9
	N-R	7.4	8.3	7.9	8.3	7.8	7.6

Tissue	Substance estimated	Experiment 2. Varying time, ± 0.2 M nicotinamide					
		Time of incubation					
		0 min.	30 min.	60 min.	120 min.	180 min.	
Brain	Nicotinamide present, DPN	9.0	8.7	7.2			
"	" " N-R	9.0	8.2	7.9			
"	" absent, DPN	8.9	0.3	0.2			
"	" " N-R	8.9	0.3	0.3			
Kidney	" present, DPN	8.5		4.6	3.2	2.3	
"	" " N-R	8.5		8.8	8.8	8.9	
"	" absent, DPN	8.4		4.0	3.1	2.1	
"	" " N-R	8.4		7.3	7.2	7.2	

* The whole vertical column represents an unincubated control; fixed at zero time.

earlier section, N-R values were almost unchanged during the course of DPN splitting by kidney. It is noteworthy that, while a slight decrease in N-R concentration was observed during DPN splitting by kidney in the absence of nicotinamide, there was no decrease when nicotinamide was present.

Evidence for DPN Nucleosidase in Kidney—The foregoing data indicate that the predominant mechanism of DPN splitting in rabbit kidney is by

pyrophosphatase action. There is also evidence of a variable amount of nucleosidase activity. It has been noted that in eight of twenty preparations incubated until 50 per cent or more of the DPN was destroyed the N-R values decreased by more than 10 per cent. Further, it has been found that under conditions in which there was a decrease in N-R value during DPN splitting this decrease could be prevented by nicotinamide (Table VI, Experiment 2).

<div align="center">TABLE VII</div>

Comparison of DPN Splitting by Kidney Homogenate, Residue, and Washed Particles

The preparation of kidney homogenate and particles (in phosphate buffer) was essentially as described under "Methods;" the residue was the sediment (not readily decanted) after centrifuging the original homogenate at 1500 R.P.M. for 5 minutes. Each fraction was adjusted to 15 ml. with phosphate buffer. The incubation mixtures were as follows: Experiments 1 and 2, 0.50 ml. of homogenate, 0.05 ml. of $MgCl_2$ (0.03 M), 0.05 ml. of NaF (0.1 M), 0.15 ml. of DPN; 1.0 ml. of residue, 0.10 ml. of $MgCl_2$ (0.03 M), 0.10 ml. of NaF (0.1 M), 0.30 ml. of DPN; 0.5 ml. of particles, 0.15 ml. of DPN, 0.1 ml. of water. The values are expressed in micromoles.

Experiment No.			Values after incubation for				
			0 min.	15 min.	30 min.	60 min.	120 min.
1	Homogenate	DPN	8.0				3.9
		N-R	8.0		7.2	7.5	7.5
	Residue	DPN	19.1				9.0
		N-R	19.1		19.6	21.8	21.8
	Particles	DPN	8.9				3.4
		N-R	8.9		8.9	8.9	9.6
2	Homogenate	DPN	8.0			4.0	
		N-R	8.0			9.2	
	Residue	DPN	14.4			9.9	
		N-R	14.4			15.5	
	Particles	DPN	8.1	6.4	5.7	4.5	
		N-R	8.1	9.2	9.5	10.0	

Since the absence of nucleosidase activity in some preparations of washed kidney particles might be due to its removal in the course of preparation, the activity of the unwashed homogenate and of the crude residue was tested. These crude kidney preparations, like the washed particles, contained no (or little) nucleosidase activity, as is shown in Table VII. It appears that the washed particles retained most of the DPN-splitting activity of the original homogenate.

<div align="center">DISCUSSION</div>

The stoichiometric recovery of adenylic acid, the absence of significant changes in the concentrations of inorganic and organic phosphate, and the

isolation of nicotinamide mononucleotide indicate that simple hydrolysis of the pyrophosphate bond is the most probable mechanism of DPN splitting by washed particles of rabbit kidney. In a system with active phosphate esterification, the data are consistent with this mechanism, since ATP is formed instead of adenylic acid.

The finding of a second pathway for enzymatic degradation of DPN and the fact that both pathways may occur in a single tissue may help clarify some questions which have arisen from assuming that DPNase is a single enzyme. Reliance on nicotinamide inhibition to preserve DPN or to evaluate DPN-splitting activity must be considered in the light of the specificity of this inhibition for nucleosidase. The failure of previous investigators to detect pyrophosphatase activity was probably related to methods of measurement. Handler and Klein (6) estimated DPN disappearance by loss of "factor V" activity for hemophilic bacteria, and Spaulding and Graham (19) relied only on the appearance of nicotinamide. Since Schlenk has shown that nicotinamide riboside satisfies "factor V" requirements (20), any splitting of DPN in which the nicotinamide-ribose moiety was preserved would not be detected by these methods.

SUMMARY

1. Two distinct enzymatic mechanisms exist for the degradation of DPN. Cleavage of the glycosidic bond between nicotinamide and the rest of the DPN molecule is catalyzed by "DPN nucleosidase." Cleavage of the pyrophosphate bond between adenylic acid and nicotinamide mononucleotide (NMN) is catalyzed by "DPN pyrophosphatase." NMN has been partially purified.

2. In rabbit brain, DPN splitting is by nucleosidase action. In rabbit kidney, pyrophosphatase action is the predominant mechanism. Nicotinamide inhibition is specific for nucleosidase.

3. Oxygen consumption and phosphate esterification by rabbit kidney particles are markedly stimulated by DPN. In a respiring system adenylic acid is recovered as adenosine triphosphate.

4. DPN disappearance is more rapid from a system in which substrate is being oxidized than from one to which no substrate has been added. Added $DPNH_2$ is split more rapidly than DPN under anaerobic conditions.

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MICROBIOLOGICAL DETERMINATION OF PHENYLALANINE IN PROTEINS AND FOODS

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Lactobacillus arabinosus (1–5), *Lactobacillus casei* (6–8), *Lactobacillus delbrueckii* LD5 (9), *Leuconostoc mesenteroides* P-60 (5, 10–12), and *Strep-*

TABLE I

Composition of Basal Medium

	gm.		mg.
Glucose	20	DL-Alanine	80
Sodium acetate (anhydrous)	12	L-Arginine hydrochloride	416
Salts A		DL-Aspartic acid	240
K₂HPO₄	1	L-Cystine	400
KH₂PO₄	1	DL-Glutamic acid + H₂O	940
Salts B	mg.	Glycine	400
MgSO₄·7H₂O	400	L-Histidine hydrochloride + H₂O	54
MnSO₄·4H₂O	20	L-Hydroxyproline	20
NaCl	20	DL-Isoleucine	50
FeSO₄·7H₂O	20	DL-Leucine	400
Adenine	100	DL-Lysine hydrochloride	300
Guanine	100	DL-Methionine	200
Uracil	100	DL-Norleucine	400
Thiamine chloride	2.0	DL-Phenylalanine*	160
Pyridoxamine dihydrochloride	0.4	L-Proline	140
Calcium pantothenate	0.4	DL-Serine	240
Riboflavin	0.4	DL-Threonine	180
Nicotinic acid	0.8	DL-Tryptophan	400
p-Aminobenzoic acid	0.4	L-Tyrosine	390
Biotin	0.01	DL-Valine	240
	γ		
Folic acid†	30‡		
Solution brought to 1000 cc. volume, pH 6.8			

K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $NaCl$, $FeSO_4 \cdot 7H_2O$

* Omitted in phenylalanine determination.
† Obtained through the courtesy of Dr. R. J. Williams, The University of Texas.
‡ 30 γ of material of "potency 5000."

tococcus faecalis (5, 13, 14) have been either proposed or used for the assay of phenylalanine. In this work the basal medium used for methionine (15) had to be modified to obtain an adequate curve with *L. mesenteroides* P-60.

TABLE II

Recovery of Phenylalanine Added to Protein Hydrolysates

Protein hydrolysate	Phenylalanine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	*per cent*
Barley, pearled	3.13	10.4	13.53	13.50	99
	6.25	10.4	16.65	16.75	101
	9.38	10.4	19.78	20.00	101
Egg, whole, dried	13.8	5.2	19.00	19.00	100
	13.8	10.4	24.20	24.50	101
	6.9	41.7	48.60	48.75	101
Ovalbumin	13.35	5.20	18.55	18.25	98
	13.35	10.40	23.75	24.00	101
	13.35	15.60	28.95	29.00	100
	6.68	41.70	48.38	48.00	99
Ox muscle	4.25	15.6	19.85	20.00	101
	8.50	15.6	24.10	24.50	102
	4.25	41.7	45.95	45.75	99

* Not corrected for moisture and ash.

TABLE III

*Phenylalanine Content of Some Proteins and Foods Determined at Different Assay Levels**

Assay level of material	Phenylalanine found									
	Ovalbumin		Brazil nut meal		Dry skim milk		Oatmeal		White rice	
γ	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cen'*
100	6.70	6.70								
200	13.50	6.75	3.40	1.70	3.10	1.55				
300	20.50	6.83								
400	26.80	6.70	6.75	1.69	6.25	1.56				
500							3.50	0.70		
600			10.50	1.75	9.50	1.58				
800			13.80	1.73	13.00	1.62				
1000							7.0	0.70	3.30	0.33
1500							10.75	0.71	.	
2000							14.75	0.74	6.90	0.34
3000									10.30	0.34
4000									13.25	0.33
Average......		6.75		1.72		1.58		0.71		0.34

* Not corrected for moisture and ash.

The new basal medium (Table I) shows pyridoxine replaced by pyridoxamine (16), arginine increased from 96 to 416 mg. per liter (17), tyrosine

increased 3-fold, norleucine increased from 120 to 400 mg., and L-methionine and L-tryptophan replaced by the DL compounds.

The requirement for extra tyrosine is interesting, as it has been shown by other workers (18–20) that there is a relationship between tyrosine and phenylalanine utilization.

EXPERIMENTAL

Leuconostoc mesenteroides P-60 was employed in the assays described.[1]
Basal Medium—The basal medium is shown in Table I.

TABLE IV

Reproducibility of Phenylalanine Content When Determined by Separate Assays*

Material	Assay 1	Assay 2	Average
	per cent	*per cent*	*per cent*
Barley, pearled.	0.63	0.61	0.62
Conarachin	4.00	4.10	4.05
Corn germ, defatted	0.79	0.79	0.79
" whole, yellow	0.63	0.61	0.62
Cottonseed flour	2.92	3.03	2.98
Edestin	5.14	5.00	5.07
Egg, whole, dried	3.45	3.24	3.35
Ovalbumin	6.68	6.75	6.72
Ox muscle	4.25	4.11	4.18
Peanut flour	3.13	2.81	2.97
Rice, white	0.34	0.32	0.33
Wheat germ, defatted	1.25	1.26	1.25
" whole	0.70	0.68	0.69
Yeast, dried, brewers'	1.67	1.50	1.58

* Not corrected for moisture and ash.

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (15, 16).

Preparation of Phenylalanine Standards—L-Phenylalanine was used to prepare the standard curve (Fig. 1). The titration values on this curve were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

Recovery of phenylalanine added to hydrolysates of barley, dried egg, ovalbumin, and ox muscle gave results well within the experimental error for this type of assay (Table II).

Table III shows values found for ovalbumin and several foods at differ-

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

TABLE V

Phenylalanine Content of Some Proteins and Foods

Percentages calculated for ash and moisture-free material.

Material	N	Phenylalan-ine	Values from literature
	per cent	*per cent*	
Arachin.....................	18.30	6.96	5.03 (21), 5.5 (22)
Casein	16.07	4.89	3.7 (2), 5.45 (5), 5.3 (8), 5.9 (9), 4.9 (11), 5.2 (12), 5.1 (13), 3.99 (21), 4.77 (23), 5.5 (24), 5.71 (25)
Coconut globulin...... ..	17.42	5.10	2.05 (26)
Conarachin.................	18.20	4.32	3.29 (21)
Cottonseed globulin...	18.00	8.13	9.1 (27)
Edestin..................	18.55	5.43	4.2 (2), 5.45 (4), 5.22 (5)
Gelatin (Bacto)........ ...	18.32	2.33	2.2 (8), 2.3 (9), 1.57 (21), 2.24 (23), 2.45 (25)
Glycinin....................	17.30	5.82	
Lactalbumin................	15.39	3.59	3.7 (8), 3.63 (21), 5.6 (22), 2.88 (23)
Ovalbumin (crystalline)......	15.98	7.17	7.9 (9), 6.0 (22), 6.18 (25)
Ox muscle............. ...	16.00	4.58	3.9 (8), 4.5 (22), 4.92 (28)
Peanut, total globulins.......	18.01	5.75	
Phaseolin (navy bean).......	16.07	8.04	
Wheat bran globulin........	17.76	4.22	
Zein..........	16.00	7.30	7.1 (22), 6.77 (25)
Barley, pearled........ .	1.86	0.68	0.45 (13)
Brazil nut meal... ..	9.03	2.06	
Corn germ, defatted .	3.93	0.96	1.37 (22), 0.71 (29)
" whole, yellow. .	2.22	0.73	0.74 (13), 0.62 (22)
Cottonseed flour.. . ..	10.36	3.40	3.56 (13), 4.4 (22), 2.69 (29)
Egg, whole, dried . .	8.11	3.71	3.0 (22), 3.09 (29), 3.24 (30)
Milk, dry, skim .	6.57	1.82	2.18 (9), 1.89 (12), 2.05 (13), 2.36 (22), 2.25 (31)
Oatmeal.. ..	2.73	0.79	0.82 (13), 1.18 (22)
Peanut flour .	10.15	3.33	3.43 (22), 1.79 (29)'
Peas, black-eyed	4.15	1.34	
Rice, white .	1.26	0.38	0.49 (22)
Rye, whole ...	1.98	0.56	0.69 (9)
Soy bean flour. ...	8.85	2.63	2.93 (9), 2.71 (13), 3.1 (22), 2.84 (29), 2.93 (32)
Wheat germ, defatted .	6.50	1.41	1.7 (22), 1.66 (29)
" whole..... .	3.07	0.78	0.96 (9), 0.81 (13), 1.07 (22)
Yeast, dried, brewers'.. .	7.71	1.79	2.12 (9), 1.73 (29), 1.97 (33)

ent assay levels. Data on the reproducibility of values found for a number of materials when determined by separate assays are given in Table IV.

The results (Table V) found for the proteins and foods[2] agree quite well with other microbiological values, but do not agree with most of the values obtained by chemical methods. This is not surprising in view of difficulties inherent in the chemical methods for this amino acid.
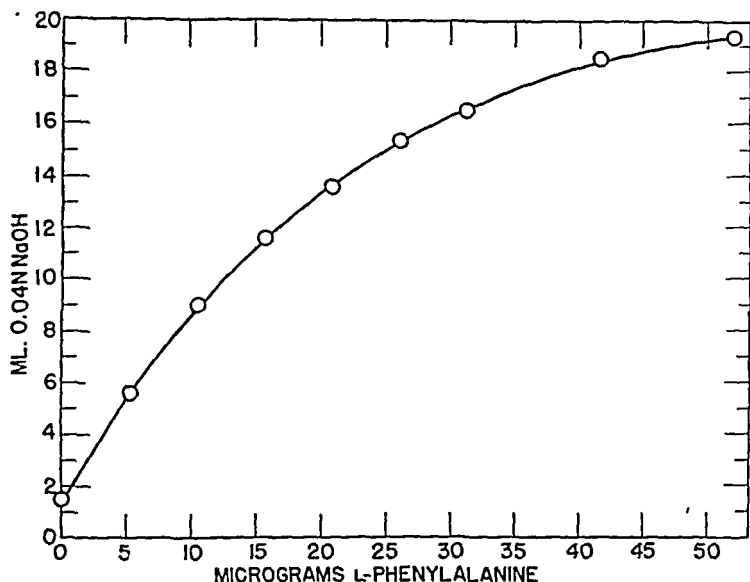
FIG. 1. Standard curve for phenylalanine

SUMMARY

A microbiological method is described for the determination of phenylalanine in proteins and foods with *Leuconostoc mesenteroides*. The results of assays on thirty-one proteins and foods agree closely with those obtained on the same materials by other microbiological methods.

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[2] The sources and preparations of the samples assayed are given in a previous publication on the determination of methionine (34).

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THE NUCLEOHISTONE OF BEEF SPLEEN

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Nucleohistones, the principal nucleoprotein components of cell nuclei, are insoluble in the moderately dilute salt solutions (0.10 to 0.15 M) which are used to extract other tissue proteins. They may, however, be extracted from cell nuclei by NaCl solutions which are much stronger (1 M or more (1)) or much weaker (less than 0.02 M (2)). While the two types of extract are chemically similar, they differ markedly in physical properties, and the question arises as to which resembles most closely the "native" nucleohistone.

When nucleohistone is dissolved in M NaCl, it shows the high and anomalous viscosity and the birefringence of flow which are characteristic of free desoxypentose nucleic acid. That the nucleic acid and histone are largely dissociated in this solvent has been shown by several investigators (1, 3, 4). The water or dilute salt extracts on the other hand ordinarily do not show birefringence of flow, and usually have a low viscosity (1, 2). When purified preparations made from these water extracts have been characterized by sedimentation and diffusion methods, they have been found to possess a degree of molecular asymmetry which, although high for a protein, is far less than that found for free desoxypentose nucleic acid (5, 6). Here the nucleic acid and histone components appear to be much more tightly bound to each other than in strong salt solutions.

These striking differences have been attributed to three different factors. The first is the degree of dissociation of the nucleic acid-histone bond, which seems to depend upon the ionic strength of the solution. The second is the specific effect which certain ions such as iodide have on the viscosity of nucleic acid solutions (7). The third factor is the action of the enzyme desoxyribonuclease which is present in all these extracts, and which depolymerizes desoxypentose nucleic acid. This enzyme is inhibited in M NaCl but is very active in dilute salt solutions (1). Water extracts of nucleohistone made before this fact was realized have, therefore, probably suffered considerable degradation (2). In fact, even when the nucleohistone is extracted in M NaCl, some enzymatic degradation may take place while the fibers are being spun in 0.14 M NaCl, especially when the enzyme is present in high concentration, as it is in the spleen (8).

In order to obtain undegraded nucleohistone it is evidently necessary

to carry out the preparation in the presence of an effective inhibitor of desoxyribonuclease. Stern and coworkers (6) have utilized arsenate for this purpose. Since desoxyribonuclease is specifically activated by magnesium ion, citrate, which binds magnesium ion, is an effective inhibitor of this enzyme (9), and should be of particular value in the isolation of undegraded nucleohistone.

In this laboratory the extraction and purification of nucleohistone from beef spleen have been carried out in the presence of citrate. Molar NaCl extracts made by this method have a much higher viscosity than extracts made without citrate. When such a M NaCl extract is dialyzed against 0.001 M citrate the nucleohistone precipitates as fibers, then redissolves to give a solution which has the same high viscosity and flow birefringence as the M NaCl solution, although the nucleic acid and histone are tightly bound to one another.

Methods

Preparation of Nucleohistone—Beef or veal spleen, obtained within half an hour of the death of the animal, was chilled on cracked ice and cubed. Approximately 100 gm. were homogenized in the Waring blendor with 250 cc. of 0.15 M NaCl or 0.05 M sodium citrate. In later experiments the spleen was cubed, frozen rapidly on dry ice, and stored in a deep freeze cabinet. The use of frozen spleen has two advantages; the tissue is brittle and easily homogenized in the blendor, and heating effects are reduced. Since no difference could be seen between the nucleohistone prepared from fresh or frozen tissue, all the experiments reported here were made on the frozen material. All operations were carried out in a cold room at 4° or less.

The first preparations, made by the method of Mirsky and Pollister (1) (in which only sodium chloride is used), had a low and variable viscosity. The procedure was then modified so as to insure the presence of an enzyme inhibitor, citrate, at all times. In the preliminary washing of the tissue the 0.15 M sodium chloride was replaced by 0.05 M sodium citrate adjusted to pH 7.0 with hydrochloric acid. The washing was repeated until the supernatant solution gave very little precipitate with 5 per cent trichloroacetic acid. The tissue was then washed once with 0.01 M sodium citrate, pH 7.0, and extracted with M sodium chloride. In the first preparations 0.05 M citrate was added to the M sodium chloride; but since solubility measurements on the purified nucleohistone showed that the presence of this amount of citrate decreased the solubility of the nucleohistone the citrate concentration was reduced to 0.01 M in later experiments. The viscous extract was cleared by centrifugation at 20,000 × g for 45 minutes, and poured into 6 volumes of water. The nucleohistone precipitated as

long fibers, which were washed quickly in 0.15 M sodium chloride and dissolved in 1.0 M sodium chloride containing 0.01 M sodium citrate, at pH 7.0. It was sometimes necessary to use a glass homogenizer to get the fibers into solution. The precipitation was repeated three or four times, and the final product kept in 1.0 M sodium chloride-0.01 M citrate solution.

In order to obtain stable solutions of nucleohistone in dilute salt, 25 cc. portions of the strong salt solution were dialyzed, with stirring, against 6 liters of 0.001 M sodium citrate. After 2 hours the nucleohistone precipitated, and after about 20 hours it redissolved completely.

High Speed Centrifugation Experiments—Nucleohistone solutions in M NaCl with 0.01 M citrate and in 0.001 M citrate were spun in the preparative rotor of an air-driven ultracentrifuge for 2 hours at $180,000 \times g$. The top 2 cc. were removed from each tube with a syringe and square tipped needle, and the samples combined. Successive lower layers were removed in the same way. Each layer was analyzed for nitrogen and phosphorus, and its ultraviolet absorption spectrum was determined.

Sodium nucleate was prepared from the nucleohistone by the method of Hammarsten (10).

Relative viscosity was measured in Ostwald viscometers at 25°.

The ultraviolet absorption spectra were determined in a Beckman spectrophotometer.

Nitrogen was measured by semimicro-Kjeldahl analysis.

Phosphorus was determined by the method of Fiske and Subbarow (11).

Purine-bound desoxypentose was determined by the diphenylamine procedure of Dische (12), with thymus nucleic acid as the standard.

Pentose nucleic acid was measured on a 5 mg. sample of sodium nucleate by the phloroglucinol reaction of von Euler and Hahn (13), with yeast nucleic acid as the standard. When the light absorption was measured in the Beckman spectrophotometer at 680 mμ, there was no interference by the desoxypentose nucleic acid.

RESULTS AND DISCUSSION

When nucleohistone was prepared from beef spleen by washing and extraction with sodium chloride in the absence of an enzyme inhibitor, the final solutions in M NaCl had a low and variable viscosity. When 0.05 M citrate was used, the product gave a steep viscosity-concentration curve, but gelled at concentrations above 100 γ of phosphorus per cc. In solutions containing 0.01 M citrate the viscosity was still high, but could now be measured at concentrations up to 150 to 200 γ of phosphorus per cc. The fact that the gel formation was more marked in 0.05 M than in 0.01 M citrate suggests that the citrate was responsible for this effect. Representative viscosity-concentration curves are shown in Fig. 1. Because these

curves rise so steeply, the relative viscosity has been plotted on a logarithmic scale. The lower part of the viscosity-concentration curve given by Mirsky and Pollister (1) for thymus nucleohistone in M NaCl has been reproduced here for comparison. These highly viscous nucleohistone preparations also showed a strong birefringence of flow. The ability of citrate to inhibit desoxyribonuclease has also been utilized by Chargaff and Zamen·
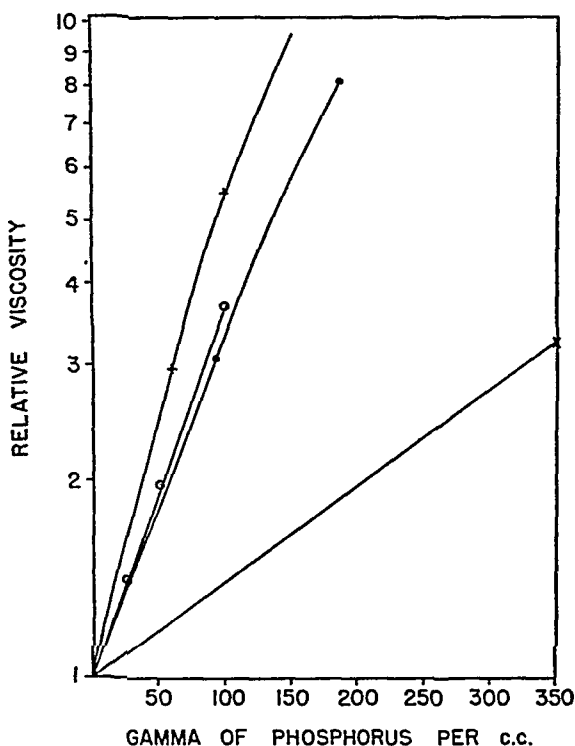
FIG. 1. The viscosity of beef spleen nucleohistone preparations made in the presence of varying amounts of citrate. All solutions contained M NaCl. ✕ no citrate, ○ 0.05 M citrate, ● 0.01 M citrate, + thymus nucleohistone, no citrate (from Mirsky and Pollister (1)).

hof (14) for the preparation of highly polymerized desoxypentose nucleic acid from yeast.

Upon dialysis against 0.01 M sodium citrate the nucleohistone precipitated in fibrous form, then redissolved completely to give a solution of unchanged nitrogen to phosphorus ratio. This solution had a viscosity almost as high as that found in M NaCl (see Fig. 2), and showed an equally strong flow birefringence. When 0.2 volume of 5.0 M NaCl was added, the nucleohistone precipitated as fibers, then redissolved to give a solution of the same

viscosity as that of a 0.001 M citrate solution of the same concentration. When this solution was allowed to stand in the cold for 1 or more days, its viscosity (measured at 25°) increased somewhat, as shown in Fig. 2.

When these preparations were dialyzed against veronal buffer for electrophoretic analysis, they precipitated and dissolved in the same way. In one experiment, made on a 0.3 per cent solution in a buffer containing 0.02
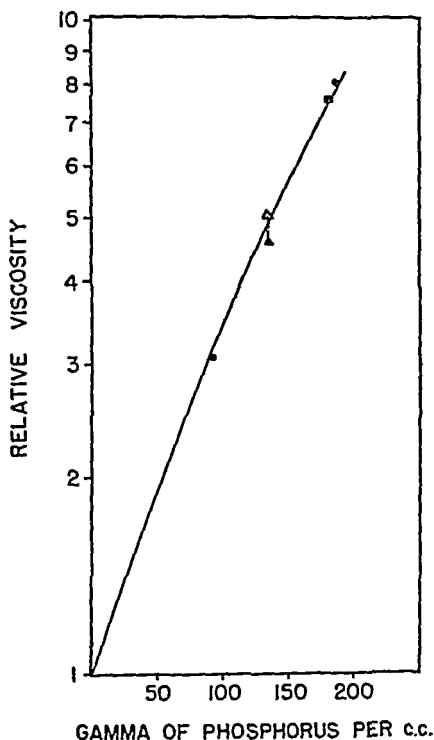
FIG. 2. The effect of the removal of sodium chloride on the viscosity of beef spleen nucleohistone. ● M NaCl + 0.01 M citrate, ■ 0.001 M citrate, ▲ 0.001 M citrate + M NaCl, △ 0.001 M citrate + M NaCl, 7 days later.

M veronal, 0.01 M NaOH, and 0.01 M NaF, at pH 7.5, a mobility of -14×10^{-5} sq. cm. per sec. per volt was found. A second experiment was made on a 0.15 per cent solution in a buffer containing 0.02 M veronal, 0.01 M NaOH, and 0.001 M sodium citrate, at pH 7.5; here the mobility was -20.1×10^{-5}. In both experiments only one boundary was seen, but since the extremely high viscosity of these solutions could prevent the separation of components of different mobility, this cannot be considered evidence of homogeneity.

When the nucleohistone was centrifuged at $180,000 \times g$ in 1.0 M NaCl

plus 0.01 M citrate about 20 per cent of the protein nitrogen remained in the upper layers, while the phosphorus was almost completely removed (see Table I). The ultraviolet absorption curves obtained on the top two layers were characteristic of protein, with maxima at 275 and minima at 250 mμ. The lower layers were extremely viscous; their nitrogen to phosphorus ratios were somewhat higher than that of the original nucleohistone and their

TABLE I

Sedimentation of Spleen Nucleohistone

Solvent	Fraction	N		P		N:P ratio		Ultraviolet absorption curve
		Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	
		γ per cc.	γ per cc.	γ per cc.	γ per cc.			
M NaCl + 0.01 M citrate	Original	865	703	255	185	3.39	3.80	Typical nucleohistone
	0–2 cc.	73	87	1.1	1.3	66	67	Histone
	2–4 "	106	129	2.5	3.4	42	38	"
	4–6 "	486	365	120	78	4.05	4.67	Nucleohistone
	6 cc. to gel		872		186		4.69	"
	Pellet		3522		1125		3.13	Between nucleohistone and nucleic acid
		Experiment 3	Experiment 4	Experiment 3	Experiment 4	Experiment 3	Experiment 4	
0.001 M citrate	Original	397	664	106	180	3.74	3.69	Nucleohistone
	0–2 cc.	7*	4.3*	1	1.2	7*	3.6*	" and extra histone
	2–4 "	10	8.7	2	1.6	5	5.4	" "
	4–6 "†		857		250		3.42	Nucleohistone
	6–7 "†	1270	2548	346	628	3.66	4.06	"

* Values too small for accurate measurement.

† Gel.

ultraviolet absorption curves also indicated a slight excess of protein. In the pellets at the bottom of the tubes, on the other hand, both chemical analysis and ultraviolet absorption indicated a greater concentration of nucleic acid over histone. These results are similar to those of Mirsky and Pollister (1), who found that when thymus nucleohistone is centrifuged in M NaCl the nucleic acid sediments more rapidly than the histone. The dialysis studies of Cohen (3) and the ultracentrifugal data of Stern and Davis (4) provide further evidence that histone and nucleic acid are partially dissociated in strong salt solutions.

In 0.001 M citrate, on the other hand, the histone was sedimented with the nucleic acid; only traces of either remained in the upper layers. The lower layers had nitrogen to phosphorus ratios and ultraviolet absorption curves very like those of the original nucleohistone solution (see Table I). These results demonstrated that in 0.001 M citrate solution the nucleic acid and histone were not appreciably dissociated. Since these nucleohistone preparations showed the same high viscosity and flow birefringence as the solutions in M NaCl and had retained their ability to precipitate in fibrous form, they must still have been composed of highly asymmetrical particles. Nucleohistone can, therefore, show the properties of a linear molecule even when the nucleic acid and histone are firmly bound.

In both the M NaCl and the dilute citrate experiments the residual chromosomes described by Mirsky and Ris (15) formed opaque pellets at the bottom of the centrifuge tubes. In Experiment 4, in 0.001 M citrate, the bottom layer was found to have a higher nitrogen to phosphorus ratio than the original nucleohistone (see Table I). This is in agreement with Mirsky's finding that the residual chromosomes have a lower nucleic acid content than the nucleohistone.

The nucleohistone in the intermediate layers, from which the residual chromosomes had been removed, had a nitrogen to phosphorus ratio of 3.4, somewhat lower than that of the original extract. This appears to be the best value for the nitrogen to phosphorus ratio of nucleohistone free of residual chromosomes. The nitrogen to phosphorus ratios obtained on the total nucleohistone extracts (after several reprecipitations) varied from 3.9 to 3.4. This variation probably means that different amounts of residual chromosomes were carried through the nucleohistone isolations.

The nucleic acid was prepared from spleen nucleohistone in the form of the sodium salt. Its ultraviolet absorption curve was similar to that of a thymus nucleate prepared by a modification[1] of the method of Gulland, Jordan, and Threlfall (16). The spleen nucleate contained 9.3 per cent phosphorus and 15.1 per cent nitrogen; the theoretical values are 9.4 and 15.9 per cent for sodium nucleate containing adenine, guanine, thymine, and cytosine in equal amounts (16). A low nitrogen content, 15.5 per cent, was also found by Gulland et al. in their thymus nucleate (16). The concentration of purine-bound desoxypentose was 105 per cent that of a thymus nucleic acid preparation of the same phosphorus content. The nucleate also contained about 2 per cent of pentose nucleic acid, an amount similar to that found by von Euler and Hahn in nucleic acid preparations from thymus and liver nuclei (13). In this laboratory small amounts of pentose have been found in thymus nucleates prepared by either the Hammarsten (10) or Gulland (16) procedures.

The nitrogen to phosphorus ratio of the sodium nucleate was 1.63, while that of the nucleohistone free of residual chromosomes was 3.42. The nucleohistone therefore contained close to 50 per cent of histone.

Whether the treatment with strong sodium chloride and with citrate has caused any irreversible changes in the nucleohistone molecule is not yet known. It has been claimed that the fibrous nature of nucleohistone in M NaCl is evidence of denaturation and that the "native nucleoprotein" isolated with the use of arsenate as an enzyme inhibitor is a relatively symmetrical molecule (6). In an attempt to answer this question we have begun the study of nucleohistone extracted from nuclei by water instead of by M NaCl.

When nucleohistone is extracted from spleen nuclei by water, the extracts are unstable even in the presence of citrate. Since the thymus contains much less desoxyribonuclease than the spleen, it was thought advisable to study water extracts of thymus nuclei washed with 0.05 M citrate. These extracts have a viscosity as high as that found in M NaCl. They do not exhibit the strong flow birefringence which is found with the strong salt extracts, but do show the "silkiness" characteristic of asymmetrical particles in suspension. When the salt concentration is increased to 0.15 M, the nucleohistone precipitates as fibers which have the spontaneous birefringence described by Cohen.[2] On repeated precipitation some preparations retain their high viscosity in water solution and their ability to precipitate as fibers, while others do not. Ahlström, von Euler, and Hahn (17) have also obtained highly viscous water extracts of nucleohistone, from thymus nuclei isolated in citric acid. The results of these experiments are far from conclusive; they do, however, suggest that the native nucleoprotein is present in the nucleus in a far more asymmetrical form than that found for the arsenate preparations (6).

SUMMARY

1. Nucleohistone preparations of high viscosity can be prepared from beef spleen by extraction with M NaCl in the presence of 0.01 M sodium citrate.

2. On dialysis against 0.001 M citrate the nucleohistone precipitates as fibers, then redissolves to give a solution of only slightly decreased viscosity, which still shows birefringence of flow. High speed centrifugation experiments show that in this solvent the nucleic acid and histone are tightly linked. Nucleohistone can, therefore, show the properties of a linear molecule even when the nucleic acid and histone are firmly bound.

3. Sodium nucleate prepared from the spleen nucleohistone resembled

[2] Cohen, S. S., personal communication.

thymus nucleate in its ultraviolet absorption characteristics and in its content of nitrogen, phosphorus, purine-bound desoxypentose, and pentose.

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THE APPLICATION OF STRUCTURAL ANALOGUES TO ENZYMATIC STUDIES*

I. STUDIES ON THE MODE OF ACTION OF BIOTIN ANALOGUES

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In recent communications (1, 2) the synthesis of a number of biotin analogues was described, and their antagonistic effects towards biotin and oxybiotin for a variety of microorganisms have been presented. Since it seems reasonable to assume that biotin functions as a constituent of an essential enzyme system, the growth-inhibiting properties of the antagonists may be due to their interference with either the biosynthesis or functioning of this system. In 1944, Winzler, Burk, and du Vigneaud (3) observed a pronounced stimulatory effect of biotin upon the fermentation rate of biotin-deficient yeast. It appeared to us that their test system would be well suited for further studies of the mode of action of our antagonists. These compounds were found to counteract the stimulatory effects upon the fermentation rate of yeast of both biotin and oxybiotin. The inhibitory effects were noted only when the inhibitors were added *prior* to the vitamin. This indicated that the antagonists function by preventing the fixation of biotin to certain cellular constituents and are without effect upon the enzymatically active form ("bound" biotin).

Methods

The experimental procedures employed in this study were patterned after those of Winzler, Burk, and du Vigneaud (3).

The basal medium employed for the growth of the yeast *Saccharomyces cerevisiae* 139 was the Hertz modification (4) of the medium described by Snell, Eakin, and Williams (5), with the omission of the casein hydrolysate. Growth on this medium was negligible but could be stimulated by either biotin or oxybiotin. The "high" biotin and oxybiotin media contained 5 mγ (millimicrograms) of biotin and 10 mγ of oxybiotin per 10 ml., respectively, and yielded maximum growth. Poor growth was obtained on the "low" biotin and oxybiotin media which contained 0.08 mγ of biotin and 0.5 mγ of oxybiotin per 10 ml., respectively. 60 ml. portions of the various media were sterilized by autoclaving at 15 pounds for 10 minutes.

After cooling, each flask was inoculated with a saline suspension of yeast grown for 24 hours of wort agar slants. After 20 hours incubation at 30°, the resulting yeast suspension was centrifuged and washed twice with the salt solution[1] used in the growth medium. The yeast was finally resuspended in the salt solution and suitable aliquots taken for the metabolic studies. The yeast concentration of these aliquots was determined by turbidity measurements in a Lumetron photocolorimeter and a calibration curve relating turbidity reading to dry weight.

Respiration (oxygen consumption) and fermentation (carbon dioxide production) measurements were made at 30° with constant volume Warburg manometers. The respiration rates were determined with air as the gas phase and 0.2 ml. of 10 per cent potassium hydroxide in the inner well. Gaseous phases of air and purified nitrogen were used for the aerobic and anaerobic measurements of fermentation, respectively. The conclusions drawn from the measurements under aerobic and anaerobic conditions were essentially the same. Since the majority of the fermentation studies were performed aerobically, only the aerobic fermentation rates will be presented. The oxygen uptake was usually neglected in calculating the aerobic fermentation rates, since it was small compared to the carbon dioxide evolution. The following constituents were always present in each Warburg flask: 0.5 ml. of the yeast suspension in the salt solution,[2] 0.5 ml. of the salt solution, and 40 mg. of glucose. The final liquid volume per flask was adjusted to 2.0 ml. with solutions of biotin, oxybiotin, aspartic acid, and inhibitors as indicated in the text. All experiments were conducted at 30° and pH 4.0. At the conclusion of each experimental run, the turbidity of the flask contents was determined. Cell counts were also occasionally performed. In no case was any significant amount of growth obtained.

<div align="center">EXPERIMENTAL</div>

Effects of Biotin and Oxybiotin on Yeast Metabolism—It has been reported (3) that the fermentation and respiration rates of a biotin-deficient yeast are considerably lower than those of a normal yeast. We have confirmed these observations and, in addition, have shown that these metabolic processes were also markedly lowered in an oxybiotin-deficient yeast, *i.e.*, yeast grown in the presence of suboptimum amounts of *dl*-oxybiotin (0.5 mγ per 10 ml). These results are presented in Table I.

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[1] Throughout this study the concentration of the salt solution added to the Warburg flasks was twice that used in the growth medium. The pH of this solution was always adjusted to 4.0 before use. Each ml. contained 5 mg. of ammonium sulfate.

[2] Amounts of dry yeast ranging from 0.5 to 5.0 mg. per flask were employed.

The "biotin effect," *i.e.* the stimulatory effect of biotin upon the fermentation rate of a biotin-deficient yeast (3), has also been observed in this laboratory. Oxybiotin could elicit a similar increase in an oxybiotin-

TABLE I

Respiration and Fermentation Rates of "High" and "Low" Biotin and Oxybiotin Yeasts*

	Low biotin	High biotin	Low oxybiotin	High oxybiotin
$-Q_{O_2}$	18	61	18	48
Q_{CO_2}	140	370	194	376

* The yeasts were grown as described in the previous section. The symbols $-Q_{O_2}$ and Q_{CO_2} represent respiration and aerobic fermentation, respectively, expressed in c.mm. per hour per mg. of dry weight. The Q values were constant during the experimental period of 2 hours. Each Warburg flask contained yeast, salt solution, and glucose as indicated in "Methods." All constituents were combined before temperature equilibration.
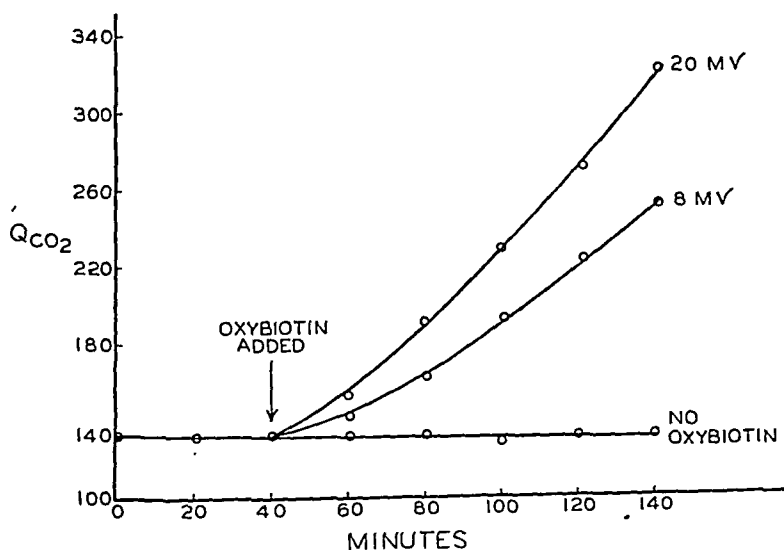
FIG. 1. The effect of oxybiotin on the aerobic fermentation of "low" oxybiotin yeast. 1.7 mg. of yeast per flask; oxybiotin added as indicated; other conditions identical with those described in Table I.

deficient yeast, as illustrated in Fig. 1 ("oxybiotin effect"). In this respect biotin was more effective than oxybiotin. A similar situation exists with regard to the growth-stimulating properties of these two compounds for yeast, oxybiotin having only 25 per cent of the gr.. ..ng potency of biotin. The fermentation-stimulating activity ..n

was also approximately 25 per cent that of biotin. It appears, therefore, that the effects upon growth correlate well with the metabolic effects, thus emphasizing the significance of the enzymatic rôle of biotin and oxybiotin in the growth process. As previously noted for the "biotin effect" (3), the stimulation by oxybiotin could be obtained only in the presence of ammonium sulfate.

Effect of Structural Analogues of Biotin on Metabolic Processes of Yeast— The three compounds depicted have been shown to inhibit the growth-promoting properties of both biotin and oxybiotin. Their ability to counteract the above "biotin and oxybiotin effects" on fermentation have now been investigated. The antagonistic activity of oxybiotinsulfonic acid is illustrated in Fig. 2.
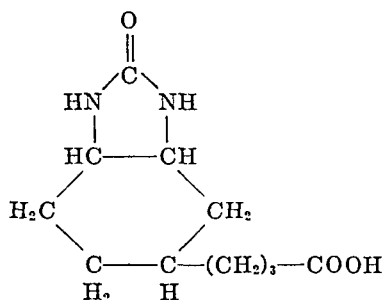
γ-(3,4-Ureylenecyclohexyl)-butyric acid[3]
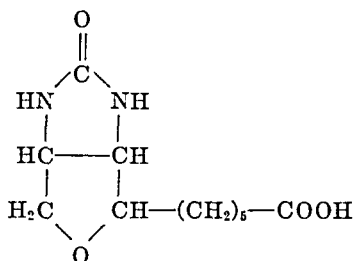
Homooxybiotin
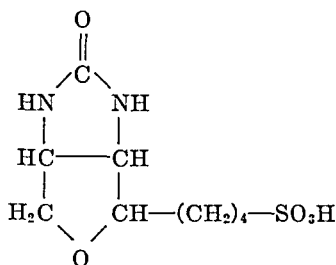
Oxybiotinsulfonic acid

It may be seen that the basal fermentation rate was not affected by 500 γ of the inhibitor (Curve I). However, the stimulatory effect of 20 mγ of oxybiotin (Curve III) could be completely abolished by 500 γ of the inhibitor added *prior* to the oxybiotin (Curve I). The stimulatory effect of biotin could also be inhibited by this analogue. The behavior of the other

[3] We are indebted to Dr. J. P. English of the American Cyanamid Company for the γ-(3,4-ureylenecyclohexyl)-butyric acid.
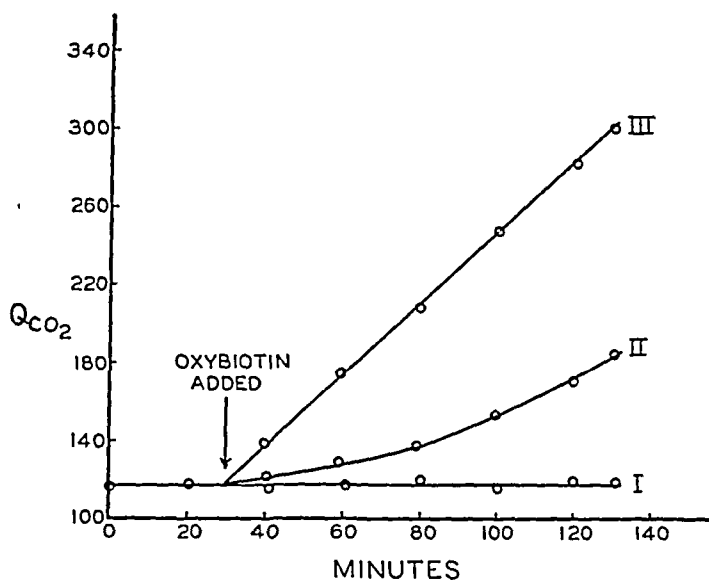
FIG. 2. Inhibition of the fermentation-stimulatory effect of oxybiotin by oxybiotin-sulfonic acid. Each flask contained 1.8 mg. of "low" oxybiotin yeast; oxybiotin-sulfonic acid added before temperature equilibration; other conditions as described in Table I. Curve I, fermentation rate under three different experimental conditions: (1) no addition, (2) 500 γ of inhibitor, and (3) 20 mγ of oxybiotin + 500 γ of inhibitor; Curve II, 20 mγ of oxybiotin + 20 γ of inhibitor; Curve III, 20 mγ of oxybiotin.

TABLE II

Inhibitory Effects of Biotin Analogues on Stimulation of Yeast Fermentation and Growth Due to Biotin and Oxybiotin

Biotin analogue	Inhibition ratio*			
	Biotin		Oxybiotin	
	Fermentation	Growth†	Fermentation	Growth†
γ-(3,4-Ureylenecyclohexyl)-butyric acid	1,000,000		75,000	
Homooxybiotin	375,000	445,000	3,750	7,400
Oxybiotinsulfonic acid	600,000	1,460,000	5,000	16,600

* Millimicrograms of inhibitor necessary to abolish completely the effect of 1 mγ of biotin or oxybiotin upon either fermentation or growth.

† Unpublished observations (Axelrod and Hofmann).

two compounds was found to be similar to that of oxybiotinsulfonic acid. The effects of the three inhibitors upon the fermentation-stimulating activities of both biotin and oxybiotin are shown in Table II. The con-

ditions of these experiments were identical with those described in Fig. 2. Varying quantities of the inhibitors were added before temperature equilibration. Biotin (1 mγ) and oxybiotin (20 mγ) were added 30 minutes *after* the zero reading. In this manner, the minimum amount of inhibitor necessary to nullify the stimulatory effects was determined. For comparison, the growth-inhibiting potency of the analogues is also reported in Table II. It is apparent that the inhibition ratios for oxybiotin are considerably smaller than those for biotin. This indicates that both the fermentation and growth-stimulating activities of oxybiotin are more easily
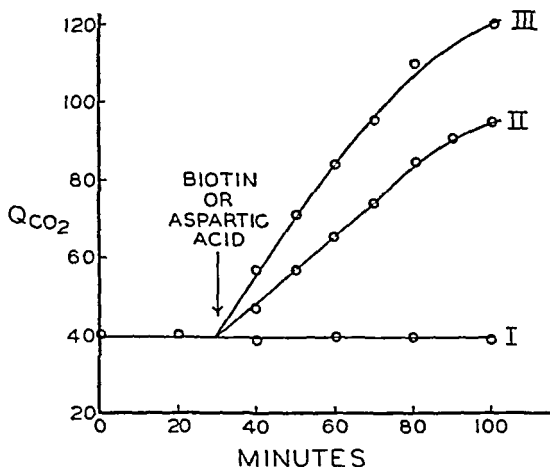
FIG. 3. Effect of aspartic acid on the fermentation of a biotin-deficient yeast. Each flask contained 3.9 mg. of yeast; biotin (5 mγ) and L-aspartic acid (800 γ) added as indicated; 7 mg. of γ-(3,4-ureylenecyclohexyl)-butyric acid added before temperature equilibration; other conditions as described in Table I. Curve I, fermentation rate under two different conditions, (1) no additions, (2) biotin + inhibitor; Curve II, fermentation rate under two different conditions, (1) aspartic acid, (2) aspartic acid + inhibitor; Curve III, biotin.

counteracted than those of biotin. The above correlation between metabolic effect and growth is again evident. The competitive nature of these inhibitions is indicated by the constancy of the inhibition ratios both for growth and fermentation over a wide range of biotin or oxybiotin concentrations.

In another set of experiments, the inhibitors were added 20 minutes *after* the biotin or oxybiotin. Under these conditions none of the inhibitors was able to prevent the progressive increase in the fermentation rates.

Stimulatory Activity of L-Aspartic Acid—As seen in Fig. 3, L-aspartic acid was capable of stimulating the fermentation of a biotin-deficient yeast in the absence of added biotin (Curve II). In the absence of ammonia,

the ability of biotin to stimulate fermentation was markedly curtailed; however, the stimulatory activity of aspartic acid was not affected. Similar results have been obtained by Winzler et al. (3). Of interest was the observation that amounts of inhibitor which were capable of completely suppressing the activity of biotin were without effect upon the stimulation by aspartic acid (Curves I and II).

DISCUSSION

In recent years many structural analogues of vitamins have been prepared and their ability to antagonize the growth-promoting effects of the parent compound has been fully investigated. It has been frequently suggested that the analogues might serve as effective tools in enzymatic studies relating to the mode of action of the vitamins. The experiments herein reported represent such a study and furnish some insight into the mechanism by which biotin analogues exert their growth-inhibiting effects. Since it is recognized that vitamins act as constituents of enzyme systems, the analogues could interfere with either the formation or the function of the active enzyme.

The observation that the antagonists counteract the "biotin effect" on fermentation only when added *prior* to the vitamin strongly indicates a successful competition with biotin in the biosynthesis of the enzymatically active complex ("bound" biotin). This complex may be regarded as a combination of either biotin or a biotin derivative with a specific protein. The inhibitors are unable to displace biotin or the biotin coenzyme from this combination and are incapable of interfering with its enzymatic function. This affords a logical explanation for the inability of the analogues to affect the basal fermentation rates of yeast where the limiting factor seems to be the amount of biotin-enzyme present in the cells. Experiments reported by Winzler et al. (3) are of interest in this connection. These authors observed that the "biotin effect" was more rapidly established when the yeast was incubated with biotin *prior* to the addition of ammonia. It seems likely that during this "pretreatment" the vitamin was converted into the enzyme essential for ammonia assimilation. The inhibition of the "biotin effect" by azide or cyanide and the failure of these compounds to inhibit the basal fermentation rate (3) could also be similarly explained. Thus the inhibitory activities of azide or cyanide might be due to their ability to inhibit certain energy-yielding processes which are necessary for the synthesis of the biotin-enzyme complex from biotin.

A relationship between biotin and aspartic acid has been indicated both for microbial growth (6, 7) and yeast fermentation (3). As shown by Winzler et al. (3) and confirmed in our experiments, aspartic acid is capable of stimulating yeast fermentation in the absence of both biotin and ammonia.

Of interest was our observation that the aspartic acid effect was not inhibited by the biotin analogues. Thus one of the functions of biotin could be related to its ability to catalyze the formation of aspartic acid in this system. The mode of action of aspartic acid in yeast fermentation remains to be elucidated.

SUMMARY

Studies on the mode of action of structural analogues of biotin have been conducted with both biotin and oxybiotin-deficient yeasts. The following observations have been made: (1) The fermentation rate of both biotin and oxybiotin-deficient yeasts was lower than that of normal yeast. This rate could be markedly accelerated by either biotin or oxybiotin. (2) A number of biotin analogues were capable of inhibiting the "biotin or oxybiotin effects" only when added *prior* to the vitamin. (3) The stimulatory effect of aspartic acid on yeast fermentation was not inhibited by the structural analogues.

It was proposed that the structural analogues exert their growth and fermentation-inhibiting effects by preventing the biosynthesis of an enzymatically active complex from biotin. One of the functions of this complex may be related to the synthesis of aspartic acid.

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THE SEPARATION AND QUANTITATIVE ESTIMATION OF PURINES AND PYRIMIDINES IN MINUTE AMOUNTS*

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The investigation of an entire series of compounds of great biological importance, the nucleic acids, nucleotides, and related substances, has been impeded considerably by the lack of specific methods for the characterization and estimation of their nitrogenous constituents. Many problems concerning the composition, metabolism, and biological functions of these substances cannot even be approached owing to the absence of sufficiently simple and widely applicable procedures.

The older methods for the determination of the total quantity of purines, reviewed by Jorpes (4), did not permit the identification of individual components. They were based, in the main, on the estimation of the nitrogen content of the purine mixtures precipitated with silver (5) or copper (6). A microprocedure founded on similar principles was described by Graff and Maculla (7). The introduction by Schmidt (8) of specific enzymes for analytical purposes represented a definite advance. More recently, Kalckar (9) made very elegant use of a combination of enzymatic and spectroscopic methods for the microestimation of individual purines. The attempt to determine the adenine content of purine mixtures by means of an adenine-deficient *Neurospora* mutant (10) has not yet given reliable results (11).

The pyrimidines have been neglected to an even higher degree. No specific methods seem to be available, and this has certainly handicapped our understanding of this important class of substances.

The procedure presented in this paper comprises essentially three steps: (*a*) the separation of the mixtures into individual components by means of chromatography on paper strips (12), (*b*) the demonstration of the number and position of separated compounds by their conversion into suitable metal salts, (*c*) the identification of the separated purines and pyrimidines

through the shapes of their characteristic absorption curves in the ultra-violet and their quantitative estimation from the extinction values.
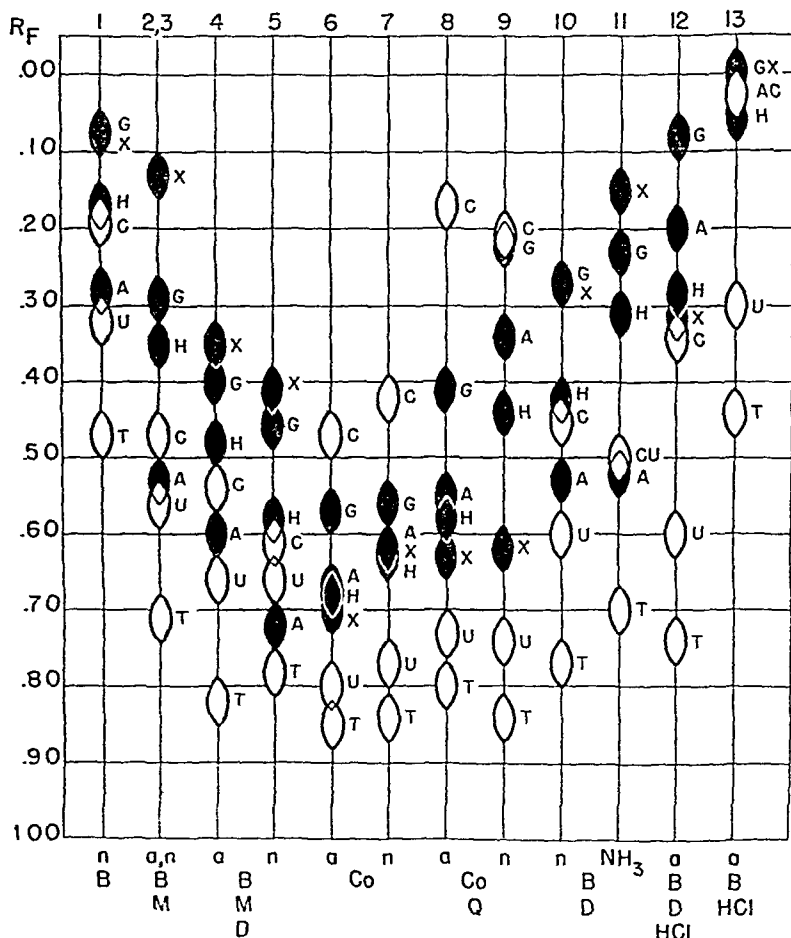
FIG. 1. Schematic representation of the position on the paper chromatogram of the purines and pyrimidines following the separation of a mixture. The numbering of the columns corresponds to the experiment numbers in Table I. *A* adenine, *G* guanine, *H* hypoxanthine, *X* xanthine, *U* uracil, *C* cytosine, *T* thymine. The conditions under which the separations were performed are indicated at the bottom. *a* acidic, *n* neutral, *B* n-butanol, *M* morpholine, *D* diethylene glycol, *Co* collidine, *Q* quinoline.

As presented here, the method applies to the separation and micro-estimation of all purines and pyrimidines normally found as constituents of nucleic acids, *i.e.* adenine, guanine, uracil, cytosine, and thymine. The

separation and identification of xanthine and hypoxanthine likewise are included, but the description of the quantitative determination of these purines, which has been achieved by similar methods, will be presented later in another connection. The procedures can doubtless be extended to other substances with characteristic absorption, such as uric acid, the pterins, etc. The following paper (13) and investigations on various desoxypentose nucleic acids to be published shortly will illustrate the application of the method to the study of nucleic acid composition. Other uses, especially for the determination of individual purines and pyrimidines in tissues and body fluids, the separation of nucleotides, etc., will be discussed at subsequent occasions.

The procedures described here should, by offering a map, as it were, of the purine and pyrimidine composition of a mixture, be particularly adapted to the detection of unexpected components. The range within which the bases can be separated and determined lies between 5 γ and 40 to 50 γ. At the optimum concentration, *i.e.* with about 20 γ of each substance, the accuracy is ± 4 per cent for the purines and even better for the pyrimidines, if the averages of a large series of determinations are considered. In individual estimations the accuracy is about ± 6 per cent. Larger amounts cannot be separated satisfactorily, because then the adsorption zones tend to become diffuse. Should the necessity arise to demonstrate very small amounts of one purine or pyrimidine in the presence of relatively large quantities of the others, it would, therefore, be advantageous to resort to a preliminary fractionation of the bases by different means before the fractions are subjected to the separation procedures discussed here.

Attention may be directed to a comparison of the efficiency of different solvent systems in effecting separation, as illustrated in Fig. 1. This may be of interest, since the choice of solvents will necessarily be governed by the type of separation that is to be performed.

EXPERIMENTAL

Material

Adenine was a synthetic preparation (14), obtained through the courtesy of Dr. M. Hoffer of Hoffmann-La Roche, Inc., Nutley, New Jersey.

Guanine (Eastman Kodak Company) was three times recrystallized from HCl as the hydrochloride and then regenerated.

Hypoxanthine was a synthetic preparation.[1] *Xanthine* (Eastman Kodak Company) was twice recrystallized from water.

Uracil and *thymine* (Schwarz Laboratories, Inc., New York) were twice recrystallized from water.

[1] We are grateful to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., Nutley, New Jersey, for this specimen.

Cytosine was prepared from uracil by the method of Hilbert and Johnson (15).[2] It was ·twice recrystallized from water.

All substances· used gave satisfactory elementary analyses.

The solvents employed were commercial preparations. *Morpholine* (Carbide and Carbon Chemicals Corporation) was rectified, b.p. 138°. *Quinoline* and *collidine* (crude), both supplied by the Koppers Company, Inc., were distilled.

The *filter paper* used for chromatography was Schleicher and Schüll, No. 597.

Selection of Solvent Systems for Separation

In Table I, the positions on the paper chromatogram of the several purines and· pyrimidines, examined in a variety of solvent mixtures, are indicated. This is done by listing the R_F values (12), *i.e.* the proportion of the distances of the starting point from the adsorbate and from the solvent front.· These values were determined at room temperature (*i.e.* at about 22°) with solutions containing only one component and were verified with mixtures.

The choice of solvent will, of course, vary with the particular problem, as can be gathered from Fig. 1, which illustrates graphically the positions of the four purines and three pyrimidines following the separation of the mixture in various solvents. Adenine and guanine may be separated from each other· in all solvent systems examined, with the exception of collidine (Experiments 6 and 7) and collidine-quinoline (Experiments 8 and 9). Xanthine, on the other hand, is best demonstrated in neutral solution in collidine-quinoline (Experiment 9). For hypoxanthine collidine-quinoline (in neutral solution), butanol, or butanol-diethylene glycol can be used. The separation, for qualitative purposes, of the four purines may be performed under the conditions expressed in Experiments 5 and 11 of Table I.

Almost all solvents examined may serve for the separation of the pyrimidines from each other. In the presence of purines, cytosine can be demonstrated in ·collidine or collidine-quinoline, uracil and thymine in butanol-HCl, both· with and without the admixture of diethylene glycol. It may be mentioned that isocytosine was found to have an R_F value in butanol very similar to that of uracil (Table I, Experiment 1).

Separation and Quantitative Estimation of Adenine and Guanine

Solutions—Because of the scanty solubility of guanine at neutrality, solutions of pH 0.8 to 1.0, usually in 0.1 N sulfuric acid, were used for the separation. Their concentration was 0.1 to 0.3 per cent with respect to

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[2] We are indebted to Dr. F. Misani for the synthesis of this substance.

each component. As a rule, 10 to 30 γ of each purine were contained in 0.01 to 0.02 cc., which was the volume serving for the individual separations. For comparative purposes, it was found important to employ similar volumes of solutions of the same degree of acidity.

TABLE I

Separation of Purines and Pyrimidines in Different Solvent Systems

Experiment No.	Mixture for separation*	Solvent system†	R_F values						
			Adenine	Guanine	Hypoxanthine	Xanthine	Uracil	Cytosine	Thymine
1	n	n-Butanol (saturated with water)	0.28	0.074	0.17	0.071	0.32	0.19	0.47
2	a	n-Butanol (3), morpholine (1), water (4)	0.53	0.29	0.35	0.13	0.56	0.47	0.71
3	n		0.53	0.29	0.35	0.14	0.55	0.46	0.73
4	a	n-Butanol (4.5), morpholine (1.5), diethylene glycol (1), water (2)	0.60	0.40	0.48	0.35	0.66	0.54	0.82
5	n		0.72	0.46	0.58	0.41	0.66	0.61	0.78
6	a	Collidine (saturated with water)	0.67	0.57	0.68	0.70	0.80	0.47	0.85
7	n		0.62	0.56	0.63	0.63	0.77	0.42	0.84
8	a	Collidine (1), quinoline (2) (mixture saturated with 1.5 parts water)	0.55	0.41	0.58	0.63	0.73	0.17	0.80
9	n		0.34	0.22	0.44	0.62	0.74	0.21	0.84
10	n	n-Butanol (4), diethylene glycol (1), water (1)	0.53	0.27	0.42	0.27	0.60	0.45	0.77
11	n		0.52	0.23	0.31	0.15	0.50	0.50	0.70
12	a	n-Butanol (4), diethylene glycol (1), 0.1 N HCl (1)	0.20	0.08	0.29	0.31	0.60	0.34	0.74
13	a	n-Butanol (saturated with 0.1 N HCl)	0.032	0.006	0.054	0.09	0.30	0.035	0.44

* a = acidic (test mixture of purines and pyrimidines dissolved in 0.1 N H_2SO_4); n = neutral (mixture neutralized on paper with gaseous NH_3 before chromatography). In Experiment 11 the separation was carried out in an NH_3 atmosphere.
† The figures in parentheses indicate volume proportions.

Separation—Paper sheets, 15 cm. wide and 50 cm. long, were divided, by ruling, into five 2.7 cm. wide longitudinal lanes. A transverse line, about 8 cm. below the top of the sheet, indicated the starting points at which, in the centers of four of the lanes, known volumes of the solution were deposited. The solutions were dispensed by means of a micro burette allowing the measurement of 0.01 cc. with an accuracy of ±1 per cent.

A micrometric burette of the Scholander type (16) or a "Gilmont ultra-microburet" (Emil Greiner Company, New York) was used. The fifth lane was left free.

Two solvent mixtures were employed in the quantitative determinations. One consisted of 4.5 parts (by volume) of *n*-butanol, 2 parts of water, 1.5 part of morpholine, and 1 part of diethylene glycol. The other mixture contained 4 parts of *n*-butanol and 1 part each of diethylene glycol and of water. When the latter solvent system was employed, the acidic purine solution was, after deposition on the paper, neutralized with gaseous ammonia[3] and the separation carried out in an ammonia atmosphere. The R_F values found with these solvent systems are included in Table I as Experiments 4 and 11. The experiments were carried out at room temperature in closed cylindrical Pyrex glass jars, 46 cm. high with an inside diameter of 21 cm. Two paper sheets were used simultaneously, their upper rims dipping, by means of suitable supports, into a trough filled with the solvent mixture. A beaker containing the same solvent was placed at the bottom of the jar. A second vessel contained N ammonia, if a NH₃ atmosphere was to be employed. The separation was terminated when the solvent front had almost reached the lower rim of the sheets, which ordinarily required about 20 hours.

Development—The paper sheets were first dried in air. The center column was then cut out, dried in an oven at 105° for 20 minutes, and sprayed with a 0.25 M solution of mercuric nitrate in 0.5 N nitric acid. The purines, thus fixed on the paper as Hg complexes, were made visible in the following manner. The sprayed paper strip was placed in a bath of 0.5 N nitric acid through which a slow stream of water was permitted to flow. The washing was considered as terminated when small paper strips, which, serving as controls, had also been sprayed with $Hg(NO_3)_2$ and put into the same bath, failed to blacken on treatment with ammonium sulfide. The chromatography strip was then passed through a solution of ammonium sulfide. Well defined black spots of mercuric sulfide indicated the position of the separated purines. As little as 5 γ could be demonstrated in this manner.

Extraction[4]—With butanol-morpholine-diethylene glycol-water as the

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[3] The purine samples that are neutralized on the paper before chromatographic separation must not contain more than 10 γ of guanine. Larger amounts of this difficultly soluble purine cannot, under neutral conditions, be recovered quantitatively, if contained in only 0.01 cc. of purine solution. For the recovery of larger amounts of guanine (compare Experiment 4 in Table III), it was found advisable to deposit two 0.01 cc. portions of solution side by side, in which case lanes 3.5 cm. wide were employed for the separation.

[4] The optical contamination of papers and extracts, for instance by contact with vapors of substances absorbing in the ultraviolet, must be avoided.

solvent, the four remaining untreated lanes were cut apart, kept for 15 minutes in an atmosphere of ammonia, in order to neutralize traces of acid, and dried in an oven at 105° until no more visible vapors were given off. With the developed center strip serving as a guide, small rectangles (usually 5 to 6 cm. long) were removed from the untreated strips at the positions of purine adsorption. Each rectangle was placed in a small test-tube (13 × 100 mm.) and kept anew in an NH_3 atmosphere for 15 minutes. The tubes were put in a water bath, maintained at 80°, and 1 cc. of absolute ether was introduced, in three portions, into each tube, in order to remove by volatilization the last traces of morpholine. After the ether had evaporated completely, exactly 4 cc. of 0.1 N hydrochloric acid were added to each tube containing adenine and 4 cc. of N hydrochloric acid to the tubes containing guanine, and the closed vessels were kept overnight at 37°. The extracts then were well mixed, cooled to room temperature, and centrifuged immediately before spectroscopy.

The use of butanol-diethylene glycol-water as the solvent rendered the extraction much simpler. The paper strips were dried in air for 3 to 4 hours and the paper segments, corresponding to the position of the separated purines, directly extracted, without further treatment, with 4 cc. portions of 0.1 N HCl (for adenine) or N HCl (for guanine).

Ultraviolet Spectroscopy—The absorption in the ultraviolet of the extracts was read, in 1 cm. quartz cells, in a Beckman photoelectric quartz spectrophotometer. The HCl extract of the corresponding paper rectangles removed from the fifth lane that had been left free, as explained above, served as the blank. Acidic extracts of filter paper themselves exhibit a low, but neither constant nor regular, absorption in the ultraviolet. For this reason, it was preferable, rather than taking the absolute extinction values at the absorption maxima (adenine at 262.5 mμ, guanine at 249 mμ) as the bases of calculation, to estimate the purine contents of the extracts from the difference in the extinction values read at the absorption maximum and at 290 mμ. For 0.001 per cent test solutions in 0.1 N HCl, *i.e.* for 10 γ of purine per cc., the difference Δ was determined as follows.

Adenine, $E_{262.5}$ 0.930; E_{290} 0.030; $\Delta = 0.900$
Guanine, E_{249} 0.737; E_{290} 0.262; $\Delta = 0.475$

In order to verify the position of the maximum, the ultraviolet absorption of the extracts was invariably also determined at 5 mμ above and below the characteristic absorption maximum of the purine in question, *i.e.* at 267.5 and 257.5 mμ for adenine, at 254 and 244 mμ for guanine. In addition, the extinction of the extracts also was measured at 300 mμ, at which wave-length the purines absorb very little light. The extinction values

found at 300 mμ should, therefore, be very low, usually between -0.010 and $+0.040$. Readings outside this range were indicative of contamination, and such extracts were discarded.

The recovery of adenine with butanol-morpholine-diethylene glycol-water as the solvent system and the readings recorded in a series of such determinations are exemplified in Table II.

The results of a typical separation of adenine and guanine by means of butanol-diethylene glycol-water are presented here as an example. A mixture of 13.6 γ of adenine and 10.08 γ of guanine was subjected to

TABLE II

*Recovery of Adenine with Butanol-Morpholine-Diethylene Glycol-Water As Solvent**

Experiment No	Adenine subjected to chromatography	Extinction at wave-length					Δ_x	$\dfrac{10\Delta_x}{\Delta}$	Adenine recovered	
		300 mμ	290 mμ	267.5 mμ	262.5 mμ	257.5 mμ				
	γ							γ	γ	*per cent*
1	19.64	0.023	0.039	0.429	0.458	0.439	0.419	4.66	18.6	95
2	19.47	0.008	0.024	0.418	0.448	0.428	0.424	4.71	18.8	97
3	19.96	0.007	0.023	0.412	0.442	0.428	0.419	4.66	18.6	93
4	19.47	0.013	0.026	0.408	0.428	0.411	0.402	4.47	17.9	92
5	19.64	0,019	0.036	0.434	0.460	0.440	0.424	4.71	18.8	96
6	19.64	0.016	0.031	0.429	0.455	0.438	0.424	4.71	18.8	96

* Δ_x is the difference in the extinction of the unknown at 262.5 and at 290 mμ, Δ the same difference for a standard solution containing 10 γ of adenine per cc. The expression $10\Delta_x/\Delta$ corresponds to the adenine concentration in 1 cc. of the unknown and, therefore, to one-fourth of the total recovered adenine.

separation. The following extinction values were recorded at different wave-lengths.

Adenine		Guanine	
300 mμ	0.005	300 mμ	0.005
290 "	0.017	290 "	0.047
267.5 mμ	0.293	254 "	0.150
262 5 "	0.311	249 "	0.164
257.5 "	0.300	244 "	0.156

The computations which follow were based on the proportion between the Δ_x values found for the unknown and the Δ values determined, as explained above, with purine solutions containing 10 γ per cc.

Adenine, $\Delta_x = 0.311 - 0.017 = 0.294$; recovered in 1 cc. ($10 \Delta_x/\Delta = 2.94/0.900$), 3.27 γ; total recovered in 4 cc., 13.1 γ; recovery, 96 per cent.

Guanine, $\Delta_x = 0.164 - 0.047 = 0.117$; recovered in 1 cc. ($10 \Delta_x/\Delta = 1.17/0.475$), 2.46 γ; total recovered in 4 cc., 9.84 γ; recovery, 98 per cent.

The results of several similar separation experiments are listed in Table III.

Separation and Identification of Adenine, Guanine, Hypoxanthine, and Xanthine

It will suffice to mention only those points in which the procedures differed from the quantitative method described above. The solutions employed (in 0.1 N H_2SO_4) were 0.1 to 0.3 per cent with respect to each of the four purines. The solvent systems used for the separation are listed in Table I.

The development of the guide strips was in all experiments carried out as described above for the quantitative estimations, with the exception of Experiments 8 and 9 (Table I) in which the paper strips were briefly washed

TABLE III

Separation of Mixtures of Adenine and Guanine by Means of n-Butanol-Diethylene Glycol-Water in NH_3 Atmosphere

Experiment No.	Adenine			Guanine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	per cent	γ	γ	per cent
1	13.6	13.8	101	10.1	10.1	100
2	13.6	12.9	95	10.1	10.4	103
3	13.6	12.9	95	10.1	9.9	98
4	27.2	25.4	93	20.1	19.0	95

with ether before being sprayed with mercuric nitrate, since quinoline interfered with the development.

For the extraction of the adsorbates, the strips were, in Experiments 1 and 10 to 13 (Table I), dried in air for 4 hours, and then divided into segments and extracted as described above. In Experiments 2 to 5 the procedures employed for the quantitative estimation with butanol-morpholine-diethylene glycol as the solvent system were followed.

In Experiments 6 to 9 (Table I), *i.e.* with collidine or collidine-quinoline,[5] it was necessary to remove the last traces of these solvents which absorb strongly in the ultraviolet. This was done by steam distillation. The paper segment was placed in a test-tube and wetted with 2 N sodium carbonate, in order to liberate the solvent. Two 0.5 cc. portions of water were then permitted to evaporate from the tube in a bath of 110°. The

[5] The use of a quartz lamp made it possible to ascertain the extent to which the strongly fluorescent quinoline sulfate had spread during the chromatography, since the separation was more complete when all purines had migrated below the acid zone.

subsequent extraction with 0.1 N hydrochloric acid was carried out in the same tubes.

In many of the separation experiments the complete absorption curves of the HCl extracts were determined, in order to check the purity of the extracted bases.

Separation and Quantitative Estimation of Uracil, Cytosine, and Thymine

Solutions—Aqueous solutions of the pyrimidines served for the separation experiments. The volumes deposited on the paper were 0.01 to 0.02 cc., containing 10 to 30 γ of each component.

Separation—*n*-Butanol, saturated with water, was employed for the separation, which required about 12 hours. Otherwise, the procedures were identical with those followed in the quantitative purine separation. The R_F values of the separated pyrimidines in several solvent mixtures are included in Table I.

Development—The paper sheet was spread out and dried in air and the center column cut out and dried in an oven at 105° for 20 minutes. It then was placed for about 30 seconds in a buffered 0.01 M mercuric acetate solution of pH 6.2. This solution was freshly prepared by mixing 1 part of 0.1 M mercuric acetate solution with 3 parts of M sodium acetate solution and 6 parts of water. The strip, after having been bathed for exactly 20 seconds in slowly renewed water, was passed through an ammonium sulfide solution. Compact spots of mercuric sulfide denoted the positions of the separated pyrimidines. The identification limits were about 5 γ for uracil and cytosine, 10 γ for thymine.

Extraction—The paper strips were dried in air for 4 hours and the 3.5 to 5 cm. long paper rectangles, cut out with the guidance of the developed strip, were each extracted with exactly 4 cc. of water in closed tubes that were kept overnight at 37°. The well mixed extracts were centrifuged before spectroscopy.

Ultraviolet Spectroscopy—The principles discussed above with respect to the estimation of the purines apply here too. The concentrations of cytosine and thymine were determined, as for the purines, from the difference in the extinction values found at the respective absorption maxima and at 290 mμ. For uracil the difference between the absorption maximum and the reading obtained at 280 mμ was used. The absorption maxima recorded for the preparations were uracil 259, cytosine 267.5, and thymine 264.5 mμ. The following values for the difference Δ were found with 0.001 per cent solutions of the pyrimidines in distilled water.

Uracil, E_{259} 0.738; E_{280} 0.148; Δ = 0.590
Cytosine, $E_{267.5}$ 0.598; E_{290} 0.053; Δ = 0.545
Thymine, $E_{264.5}$ 0.626; E_{290} 0.081; Δ = 0.545

As was explained before with regard to the purines, additional absorption readings at 5 mμ above and below the respective maxima and also at 300 mμ served to ascertain the purity of the extracts. The readings recorded in a typical separation of uracil, cytosine, and thymine are exemplified below. Table IV summarizes the results of five separation experiments.

TABLE IV

Separation of Mixtures of Uracil, Cytosine, and Thymine

Experiment No.	Uracil			Cytosine			Thymine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*
1	24.3	23.1	95	24.8	23.9	96	24.8	24.5	99
2	25.0	23.7	95	25.0	24.5	98	25.0	23.6	95
3	24.5	24.5	100	24.3	24.6	101	24.5	23.1	94
4	11.9	11.9	100	10.7	10.5	98	11.9	11.9	100
5	11.8	11.4	97	10.5	9.9	94	11.8	11.2	95

TABLE V

Extinction Values for Eluates of Separated Pyrimidines

Uracil		Cytosine		Thymine	
Wave-length	E	Wave-length	E	Wave-length	E
mμ		*mμ*		*mμ*	
300	0.004	300	0.003	300	−0.003
280	0.046	290	0.014	290	0.016
264	0.210	272.5	0.150	269.5	0.172
259	0.222	267.5	0.157	264.5	0.178
254	0.212	262.5	0.153	259.5	0.174

A mixture containing 11.9 γ of uracil, 10.7 γ of cytosine, and 11.9 γ of thymine was subjected to a chromatographic separation. The readings of the extinction values recorded for the eluates (4 cc.) of the separated pyrimidines are shown in Table V.

The computations, based on the proportion between the Δ_x values found for the unknown and the Δ values found for pyrimidine solutions containing 10 γ per cc., follow here.

Uracil, $\Delta_x = 0.222 - 0.046 = 0.176$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.76/0.590$), 2.98 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

Cytosine, $\Delta_x = 0.157 - 0.014 = 0.143$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.43/0.545$), 2.62 γ; total recovered in 4 cc., 10.5 γ; recovery, 98 per cent.

Thymine, $\Delta_x = 0.178 - 0.016 = 0.162$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.62/0.545$), 2.97 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

SUMMARY

Mixtures containing minute amounts of purines (adenine, guanine, hypoxanthine, xanthine) and pyrimidines (uracil, cytosine, thymine) were separated in a variety of solvent systems. The method developed for this purpose, which makes use of paper chromatography, permits not only the demonstration of the individual components by their conversion into mercury salts, but also their identification and quantitative estimation by means of ultraviolet spectroscopy. Amounts ranging from 5 to 40 γ of adenine, guanine, uracil, cytosine, and thymine thus were separated and determined quantitatively.

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THE COMPOSITION OF THE PENTOSE NUCLEIC ACIDS OF YEAST AND PANCREAS*

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The historically understandable attempts to simplify the problem of nucleic acid structure by the formulation of the tetranucleotide hypothesis have found their classical exposition in Levene's monograph of 1931 (1). The same tendencies are expressed, less precisely, in statements found in many text-books according to which the nucleic acid unit, having a molecular weight of about 1200, contains four different bases (two purines and two pyrimidines) in equimolecular proportions. With the growing recognition of the complex and macromolecular character of the nucleic acids the necessity for the postulation of these hypothetical units vanished and it became possible to consider nucleic acids as substances comparable to the proteins in intricacy and, perhaps, even in specificity.

Our present knowledge of the structure of nucleic acids has been reviewed repeatedly in the recent past (2–4). Other relevant aspects also have been considered (5, 6).

The method for the quantitative estimation of individual purines and pyrimidines in mixtures of these bases, presented in the preceding paper (7), has made possible a new approach to the study of the composition of nucleic acids. The present communication describes the application of these procedures to a study of the distribution of the nitrogenous constituents of the pentose nucleic acids of yeast and pig pancreas and includes a consideration of aspects related to this problem, such as the mechanisms of the acid hydrolysis of these compounds. Other accounts, to be submitted shortly, will deal with the composition of desoxypentose nucleic acids. The results reported here will, later in this paper, be correlated with the findings of previous workers.

EXPERIMENTAL

Material

Ribonucleic Acid of Yeast—The purification procedure used was a modification of that of Fletcher *et al.* (8).   15 gm. of yeast nucleic acid (Merck)

were dissolved in 400 cc. of water by the addition of 15 cc. of 2 N ammonia. The filtered solution (pH 6.2) was dropped, with mechanical stirring, into 860 cc. of 95 per cent ethanol containing 5 cc. of concentrated HCl. The precipitate, collected by centrifugation, was washed with 125 cc. of 66 per cent ethanol and redissolved in 250 cc. of water and 16 cc. of 2 N ammonia. The precipitate obtained from the filtered solution with 500 cc. of alcohol and 4 cc. of concentrated HCl was suspended in 25 cc. of water and subjected to dialysis against running water (14 hours) and several changes of ice-cold distilled water (20 hours). The ribonucleic acid was collected, washed with 66, 98, and 100 per cent ethanol and with ether, and dried *in vacuo* over P_2O_5; it weighed 7.2 gm. For analysis, the substance was dried in a high vacuum at 60° for 3 hours and weighed out under exclusion of moisture.

Found, N (Dumas) 15.3, P (Pregl-Lieb) 8.0

This nucleic acid showed the characteristic absorption spectrum in the ultraviolet (pH 6.5). For the maximum at 257.5 mμ an ϵ(P) value of 9800 was found; at the minimum of 229 mμ ϵ(P) was 4200 (for definitions, see (9)).

Pentose Nucleic Acid of Pig Pancreas[1]—The preparation of this material followed in all essentials the procedures described by Jorpes (10, 11) and by Levene and Jorpes (12). The free pentose nucleic acid gave no protein tests and contained only a small amount (3 per cent) of desoxypentose nucleic acid (diphenylamine reaction). The substance was, for analysis, dried as described above.

Found, N (Dumas) 15.4, P (Pregl-Lieb) 7.9

The absorption maximum of this preparation (pH 6.3) was at 256 mμ with an ϵ(P) of 9800; the corresponding value for the minimum at 228 mμ was 4200.

Ribose Nucleotides—Adenylic acid was prepared from commercial yeast nucleic acid (Schwarz Laboratories, Inc., New York) in the crystalline state by the recent method of Buell (13). A few modifications were introduced, which included the use of 0.5 N alkali for 24 hours at 37° for the cleavage of the nucleic acid, and the repeated precipitation of the nucleotide as the lead salt before the recrystallization of the free adenylic acid from water. The air-dried white crystalline substance contained N (Dumas) 18.54, P (Pregl-Lieb) 8.45 per cent. It lost 2.6 per cent of its weight when dried for 2 hours at 109° *in vacuo*.

Basic sodium guanylate was prepared as a white crystalline powder from

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[1] We are indebted to Dr. F. Misani for help with this preparation.

the potassium acetate complex of guanylic acid, collected in the course of the preparation of adenylic acid, by the method of Steudel and Peiser (14). The material, dried at 77° *in vacuo*, contained N (Dumas) 13.27, P (Pregl-Lieb) 6.16 per cent.

Cytidylic acid (Preparation 1) was prepared from the acid hydrolysate of commercial yeast nucleic acid by the fractional crystallization of the brucine salts according to Levene (15, 16). The nucleotide, recrystallized from 35 per cent ethanol and dried *in vacuo* at 65°, contained N 12.3, P 9.0 per cent, and had a rotation of $[\alpha]_D^{27} = +50.3°$ (0.1 per cent solution in water). Another specimen of cytidylic acid (Preparation 2) was obtained through the courtesy of Dr. S. J. Thannhauser and Dr. G. Schmidt. It contained, after recrystallization from 35 per cent ethanol, N 12.4, P 9.5 per cent, and had $[\alpha]_D^{27} = +50.8°$ (0.1 per cent solution in water). Levene (16) found for this nucleotide a rotation of $[\alpha]_D^{50} = +48.5°$.

Quantitative Estimation of Purines in Nucleic Acids

The nucleic acid was dried in a high vacuum at 60° for 3 hours and 5 to 8 mg. of the preparation, weighed on a micro balance, were placed in a small Pyrex bomb tube (160 × 5 mm.); 0.5 cc. of N sulfuric acid was added and the sealed tube heated for 1 hour at 100° in a boiling water bath. The clear solution was allowed to cool and was transferred, by means of a long capillary pipette, into a 1 cc. volumetric flask. The tube walls were rinsed three or more times with a few drops of 0.1 N H_2SO_4. The washings served to bring the hydrolysate up to volume. When the solvent system used for the subsequent purine separation consisted of *n*-butanol, morpholine, diethylene glycol, and water (7), the undiluted hydrolysate in the volumetric flask was first adjusted to pH 0.8 to 1 by means of a few drops of 30 per cent aqueous NaOH and then brought to a volume of exactly 1 cc. with 0.1 N H_2SO_4, the washings being used for this purpose. The pH adjustment was controlled by dipping the tip of a sliver of hydrion paper (pH 0 to 1.5) into the solution. When morpholine was omitted from the solvent mixture (7), no alkali was added and the solution was brought up to volume directly with 0.1 N H_2SO_4.

Several 0.01 to 0.02 cc. portions of this solution, dispensed on paper, as described in the preceding publication (7), by means of an accurate micrometric burette, served for parallel separations and estimations. As a rule, six determinations were carried out simultaneously with the same hydrolysis fluid. The procedures used for the development of the guide strip, which indicated the number and positions of the separated purines, and for the extraction and the identification and estimation of the components by spectroscopy in the ultraviolet followed exactly the methods described

before (7).[2]   In all analyses, a drop of the hydrolysate and a drop of a purine test mixture of known composition were chromatographed side by side on a separate paper sheet, in order to compare the positions of the adsorbates on the chromatogram and thereby to verify the identification of the separated components.

Quantitative Estimation of Pyrimidines in Nucleic Acids

A portion, weighing between 15 and 25 mg., of the nucleic acid, that had been dried in a high vacuum at 60° for 3 hours, was weighed exactly into the special vessel shown as C in Fig. 1.   Absolute methanol (0.5 to 1 cc.) was added and dry HCl gas passed, by means of a capillary, through the suspension with complete exclusion of moisture.   A copper spiral through which cold water circulated converted the neck of the flask into a reflux condenser.   (The experimental arrangement is illustrated in Fig. 1.) Within about 30 minutes the nucleic acid dissolved in the methyl alcohol, which became warm, and the purine hydrochlorides began to precipitate. The passage of HCl gas then was continued for 3 to 5 hours while the mixture was kept at 50°.   After being chilled overnight with rigorous exclusion of moisture, the closed reaction vessel was centrifuged.   The supernatant was quantitatively transferred to a bomb tube (220 mm. long, inside diameter 65 mm., outside diameter 85 mm.) by means of a capillary siphon which was actuated by suction.[3]

The yellowish methanol solution was evaporated at about 45° under a nitrogen current and the evaporation was continued, with the addition of small portions of fresh methanol, until the alcohol vapors carried almost no acid.   About fifteen evaporations were, as a rule, necessary.   The residue was dried overnight *in vacuo* over $CaCl_2$ and KOH.   After the introduction of 0.5 cc. of concentrated formic acid (98 to 100 per cent, Eastman Kodak), the bomb tube was sealed and heated at 175° for 2 hours.   It then

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[2] It might be pointed out that it is possible to ascertain the presence on the chromatogram of minor purine or pyrimidine components whose concentrations are insufficient to permit their direct demonstration as Hg salts.   As the relative positions of the individual purines and pyrimidines on the chromatogram are known (7), the extract of a segment, removed from the paper strip at the location of the suspected substance, may serve for the spectroscopic examination.   In this manner, the pentose nucleic acid of pancreas was tested for thymine, but none was found.

[3] It was found preferable to omit the washing of the purine hydrochloride sediment, since this resulted invariably in the contamination of the pyrimidine fraction by purines, even when cold methanol saturated with gaseous HCl served as the wash fluid.   The precipitated purine hydrochlorides, dissolved in 0.1 N H_2SO_4 and brought up to a known volume, may be subjected to separation by chromatography and estimation.   The results, however, were not as constant as when the purine hydrolysis was carried out with N H_2SO_4, as described in the preceding section.

was chilled to 0° and, because of inside pressure, opened with all necessary precautions.

To the dark brown hydrolysate 2 to 3 drops of about 40 per cent NaOH solution were added, which effected the flocculation of the pigment and the clarification of the solution.[4] The tube was centrifuged, the light yellow supernatant transferred to a 1 cc. volumetric flask, and the centrifugation
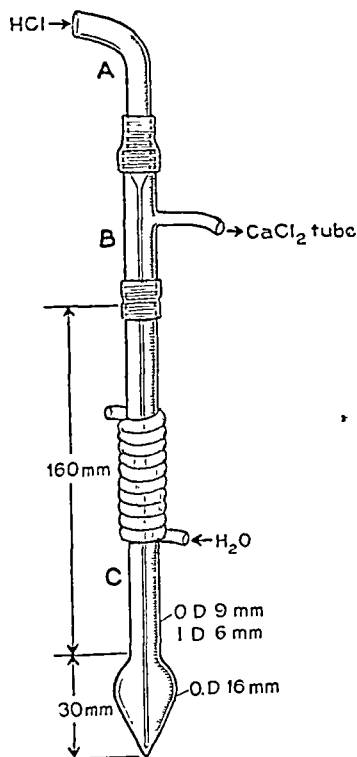
FIG. 1. Arrangement for the hydrolysis of very small amounts of nucleic acid The capillary tube *A* is connected through the adapter *B* with the reaction vessel *C*.

residue washed, at least three times, with 0.1 to 0.2 cc. portions of warm water. The centrifuged washings were used to bring the volume of the hydrolysate up to 1 cc. Several, usually six, exactly measured 0.01 to 0.02 cc. portions of this solution were deposited on the paper sheets serving for the chromatographic separation and were neutralized with gaseous ammonia. The separation with *n*-butanol-water as the solvent, the development, the extraction, and the identification and quantitative determination

[4] When insufficiently clarified brown solutions are subjected to chromatography, dark tongues form on the paper which interfere with the quantitative spectroscopy.

of the separated pyrimidines were carried out by the method previously described (7).

Control Experiments

Resistance of Pyrimidines to Acid Treatment—It is well known that the liberation of pyrimidines from nucleic acids requires an extremely drastic treatment, *e.g.* autoclaving at 175° for several hours with 20 per cent HCl (17) or 25 per cent H_2SO_4 (18). It appeared of interest to take advantage of the ease with which changes in the composition of pyrimidine mixtures can be followed with the new chromatographic method (7) by studying the effect of various acids on a mixture of uracil, cytosine, and thymine.

TABLE I

Resistance of Pyrimidines to Treatment with Strong Acid

A mixture of pyrimidines of known concentration was dissolved in the acids indicated below and heated at 175° in a bomb tube. The concentration shifts of the individual pyrimidines were determined through a comparison of the recoveries of separated pyrimidines before and after the heating of the mixture.

Experiment No.	Acid	Heating time	Concentration shift, per cent of starting concentration		
			Uracil	Cytosine	Thymine
		min.			
1	HCl (10%)	90	+62	−63	+3
2	10 N HCOOH + N HCl (1:1)	60	+3	−5	0
3		120	+24	−19	0
4	HCOOH (98 to 100 %)	60	0	−1	−2
5		120	0	+2	+1

The acids examined were 10 per cent hydrochloric acid, a mixture of equal parts of 10 N formic and N hydrochloric acids (19), and, finally, pure formic acid (98 to 100 per cent).

The original solution that served for the experiments contained 49.1 mg. of uracil, 50.4 mg. of cytosine, and 44.4 mg. of thymine in 10 cc. of 10 per cent HCl. A 2 cc. aliquot of this solution was neutralized with concentrated aqueous NaOH and diluted with water to an exact volume of 5 cc. Several 0.01 cc. portions of this neutral solution were subjected to chromatographic separation on filter paper with *n*-butanol-water as the solvent and to quantitative spectroscopic examination of the separated components (7). Another 2 cc. aliquot of the original test mixture was heated in a bomb tube for 90 minutes at 175°, neutralized and diluted to 5 cc., as described before, and likewise subjected to quantitative separation. The experiments with other acids were carried out in a similar manner. The results, assembled in Table I, indicate the instability of cytosine,

which was to a large extent converted to uracil, in acids other than formic acid; uracil and thymine resisted the acid treatment.

Hydrolysis of Yeast Ribonucleic Acid with Strong HCl—Following the liberation of purines by hydrolysis with gaseous HCl in dry methanol and the concentration of the supernatant from the purine hydrochlorides to dryness, as described above, the pyrimidine nucleotide residue was heated, in a sealed tube, with 0.3 cc. of 10 per cent hydrochloric acid at 175° for 2 hours. The pyrimidines were separated in the usual manner. Under these conditions the nucleic acid yielded 2.1 per cent of cytosine, 7.0 per cent of uracil. A comparison with the quantitative estimations summarized in Table VI will show that the hydrolysis with strong mineral acid produced an enormous shift in the relative proportions of the two pyrimidines, although the total amount recovered was nearly the same. In the HCl hydrolysate the mole proportions of pyrimidine to phosphoric acid were cytosine 0.073, uracil 0.242; the cytosine N amounted to only 5.2, the uracil N to 11.4 per cent of the nucleic acid N; the molar cytosine to uracil ratio was 0.3.

Hydrolysis of Yeast Ribonucleic Acid with Formic and Hydrochloric Acids— The following experiments were designed to exclude the possibility that the attacks on the nucleic acid by formic and by hydrochloric acids were directed against different groupings and were selective with respect to the proportions of liberated pyrimidines. From 48.052 mg. of yeast ribonucleic acid (dried in a high vacuum) the purines were split off as the hydrochlorides, in the manner described, by means of methanolic HCl. The methanolic supernatant was evaporated and freed of HCl and the residue transferred to a 2.5 cc. volumetric flask and dissolved in methanol. Two 1 cc. portions of this solution, designated Solutions A and B, were subjected to hydrolysis. Solution A was taken to dryness, the residue heated with 1 cc. of concentrated formic acid for 2 hours at 175° in a sealed bomb tube, and the pyrimidine distribution determined in the usual manner. The evaporation residue of Solution B was similarly treated with formic acid. The hydrolysate was evaporated (at the end with frequent additions of absolute ethanol) under nitrogen at 45° and the residue again subjected to hydrolysis in a sealed tube, this time with 1 cc. of 20 per cent HCl, at 175° for 2 hours. The following results on the pyrimidine distribution again confirmed the destructive effect of mineral acids on cytosine, but ruled out the assumption of a differential action of formic and hydrochloric acids: Solution A, cytosine 6.8, uracil 2.4, total pyrimidines 9.2 per cent; Solution B, 3.5, 5.7, and 9.2 per cent, respectively.

Hydrolysis of Nucleotides—3.264 mg. of *adenylic acid* were hydrolyzed with N sulfuric acid at 100° for 1 hour as described above. Aliquots corresponding to 32.64 γ of the nucleotide yielded, when chromatographed in

n-butanol-diethylene glycol-water, 11.1 γ of adenine (95 per cent of the amount expected from the N content). *Sodium guanylate*, similarly treated, gave, per 29.08 γ of the nucleotide, 8.34 γ of guanine (100 per cent).

The hydrolysis of *cytidylic acid* was studied with the two preparations mentioned before. With Preparation 1 the liberation of phosphoric acid and of cytosine by treatment with concentrated formic acid for various periods was followed and compared with the behavior of yeast ribonucleic acid under analogous conditions. Both the nucleotide and the nucleic acid were subjected to the operations necessary for the removal of the purines, the hydrolysis with formic acid at 175° in a bomb tube, and the determination of the pyrimidines in the manner described. At the same

TABLE II

Liberation of Pyrimidines and of Phosphorus from Yeast Ribonucleic Acid and from Cytidylic Acid by Heating with Concentrated Formic Acid

Duration of heating at 175°	Yeast ribonucleic acid		Cytidylic acid		
	Inorganic P	Total pyrimidines (cytosine + uracil)	Inorganic P	Cytosine	Cytosine N
min.	*per cent of total P*	*per cent*	*per cent of total P*	*per cent*	*per cent of nucleo- tide N*
0	6		0		
30	83				
60	85		76		
90	100	8.2	100	22.7	70
120	100	9.2	100	25.8	80
150				25.6	79
210		9.1			
300		9.2			

time, the total and the inorganic phosphorus contents were estimated colorimetrically in dilutions of the hydrolysates. The results will be found in Table II. Another set of experiments with cytidylic acid (Preparation 2), reproduced in Table III, was designed to compare the effects of 90 and 99 per cent formic acids and of very strong hydrochloric acid. All hydrolyses were performed in the customary manner in bomb tubes at 175°. It will be seen (Experiment 1a) that the action of fresh acid on a hydrolysate produced little additional effect. The behavior of cytidylic acid preparations toward hydrolysis obviously will require additional study. A glance at Tables II and III will show that the recovery of cytosine amounted in no case to more than about 80 per cent of the nucleotide nitrogen. It was even lower with strong hydrochloric acid as the hydrolyzing agent when, as expected, a portion of the cytosine was converted to uracil (Experiment

3 in Table III). The reasons for this refractoriness of cytidylic acid are not yet clear.

Composition of Yeast Ribonucleic Acid

Purines—The only purines encountered on the chromatograms were adenine and guanine. The absorption maximum of adenine (in 0.1 N HCl) was at 263 mμ, that of guanine (in N HCl) at 249 mμ. A series of estimations, each representing the average of at least six parallel determinations, is assembled in Table IV. The average of all adenine values reported is 9.1 per cent, that of all guanine values 10.2 per cent. If the value for guanine found for Hydrolysate 13 (Table IV), which appears rather out of range, is disregarded, the guanine average becomes 10.0 per

TABLE III

Hydrolysis of Cytidylic Acid

Experiment No.	Acid	Duration of heating at 175°	Pyrimidines found			
			Cytosine	Cytosine N	Uracil	Uracil N
		hrs.	*per cent*	*per cent of nucleotide N*	*per cent*	*per cent of nucleotide N*
1	99% HCOOH	2	23.8	73	0	0
1a*	99% "	4	25.4	78	0	0
2	90% "	2	26.0	79	0	0
3	20% HCl	2	16.6	51	6.5	13

* An aliquot of the hydrolysate obtained in Experiment 1 was evaporated to dryness under N$_2$ at 45° and the residue heated with a fresh portion of concentrated formic acid for 2 hours. Only traces of absorbing material were found at the chromatographic position of uracil.

cent. The experiment with Hydrolysate 22 is for several reasons considered as the most reliable; it will be seen that the figures reported for it differ only very slightly from the averages computed for the entire series. When adenine in Hydrolysate 4 and guanine in Hydrolysate 22 each were determined with the use of two different solvent systems, the results were in very good agreement.

Pyrimidines—Cytosine and uracil, the latter a relatively minor component, were the pyrimidines found in the hydrolysates. The absorption maximum for cytosine was at 268 mμ, that for uracil at 259 mμ (solutions in water). The results of several determinations are summarized in Table V. The average value for cytosine was 6.7, that for uracil 2.3 per cent. These figures, it is felt, require an upward correction of 5 per cent. As was pointed out above (see also foot-note 3), it was found advantageous to omit the washing of the purine hydrochlorides that precipitated in the course of the hydrolysis with methanolic HCl, in order to avoid the contamination of

the pyrimidine fraction. This involved, of course, a small loss in pyrimidines owing to the retention of some pyrimidine nucleotide by the purine sediment. For an estimate of the extent of this loss, hydrolysates of thymus nucleic acid were employed, since the position of thymine on the chromatogram rendered it the pyrimidine least affected by contamination with purines (7). It was thus found, in comparative hydrolysis experiments, that approximately 5 per cent of the thymine was retained in the

TABLE IV

*Purine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Solvent system		Adenine	Guanine
			per cent	*per cent*
1	n-Butanol-morpholine†		9.7	
2	"		8.6	
4	"		9.5	
4	n-Butanol-diethylene glycol-morpholine		9.5	
7	"	"	9.2	
10	"	"	9.9	
13	"	"	9.6	12.2
14	"	"	8.6	9.7
21	"	"	8.1	9.3
22	"	"	9.0	10.1, 10.4‡
24	"	"		10.3
25	"	"		10.0
27	"	"	8.4	9.9

* Each value represents the average of at least six parallel determinations (concordant within a range of 5 per cent) on the same hydrolysate.

† This solvent system, described in the preceding paper (7) for qualitative separations, may be used for the estimation of adenine but not of guanine. The procedures are the same as for the solvent containing diethylene glycol.

‡ In this determination n-butanol-diethylene glycol (in NH_3 atmosphere) was employed as the solvent.

purine hydrochloride precipitate. For this reason, the pyrimidine figures given in Tables VI and VIII, which summarize the distribution of the nitrogenous components of pentose nucleic acids, were corrected by this factor.

Proportions and Balances—Table VI provides a survey of the distribution of purines and pyrimidines in the yeast ribonucleic acid. The fact that the purines or pyrimidines liberated by the hydrolysis of a nucleotide contain 1 hydrogen atom more than the corresponding radicals present in the uncleaved compound was left out of consideration, since the contributions from this correction would have been negligible. It will be seen that the molar proportions (taking uracil as 1) and the mole per mole of phosphorus

ratios of the nitrogenous constituents rule out the existence of a regular tetranucleotide. It is of course understood that the computations presented here are not at all dependent upon any particular assumption regarding the structure of the nucleic acid analyzed. That the recoveries in terms of total nitrogen and of total phosphorus were closely similar, but

TABLE V

*Pyrimidine Content of Yeast Ribonucleic Acid**

Hydrolysate No.	Cytosine	Uracil
	per cent	*per cent*
32	†	2.1
34	7.4	2.3
35	6.5	2.4
36	6.1	2.3
38	6.8	2.4

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. In all separations *n*-butanol (saturated with water) served as the solvent.

† The absorption spectrum of the cytosine solution, isolated in this experiment, indicated contamination.

TABLE VI

*Yeast Ribonucleic Acid; Proportions and Balances**

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	Purine N / Pyrimidine N	Mole per mole P	P accounted for	Moles per 4 moles P	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*		
Adenine....................	9.1	4.72	30.9		0.261	26.1	1.04	3.2
Guanine....................	10.0	4.63	30.3		0.256	25.6	1.02	3.1
Cytosine.....	7.0	2.65	17.3		0.244	24.4	0.98	3.0
Uracil.....................	2.4	0.60	3.9		0.083	8.3	0.33	1.0
Total nucleic acid.........			82.4	2.9		84.4		

* The nucleic acid preparation contained N 15.3, P 8.0 per cent.

did not quite reach 100 per cent, may be significant, as will be pointed out later.

Composition of Pancreas Pentose Nucleic Acid

As was the case with the yeast nucleic acid discussed immediately above, adenine, guanine, cytosine, and uracil were the four nitrogenous constituents encountered on the chromatograms. Tests carried out with extracts

collected in the region of thymine adsorption (compare foot-note 2) failed to indicate the presence of this pyrimidine. The series of estimations reproduced in Table VII revealed an average content of adenine 5.7, guanine 15.5, cytosine 5.5, uracil 1.2 per cent. Guanine, therefore, was by

TABLE VII

Purine and Pyrimidine Content of Pancreas Pentose Nucleic Acid*

Hydrolysate No.	Adenine	Guanine	.	Cytosine	Uracil
	per cent	*per cent*		*per cent*	*per cent*
1	6.0	15.4			
2†	5.5	16.0			
3	5.6	15.2			
5				5.3	1.5
6				5.9	1.1
7				5.2	1.0

* Each value represents the average of at least six parallel determinations (concordant within 5 per cent) on the same hydrolysate. The purine determinations were all carried out with *n*-butanol-diethylene glycol-morpholine as the solvent; for the estimation of pyrimidines *n*-butanol was employed.

† In this experiment the duration of hydrolysis was doubled to 2 hours.

TABLE VIII

Pancreas Pentose Nucleic Acid; Proportions and Balances*

Compound	Content in nucleic acid	Nitrogen in nucleic acid	N accounted for	$\dfrac{Purine\ N}{Pyrimidine\ N}$	Mole per mole P	P accounted for	Molar proportions
	per cent	*per cent*	*per cent of nucleic acid N*			*per cent of nucleic acid P*	
Adenine.............. .	5.7	2.95	19.2		0.166	16.6	3.6
Guanine...	15.5	7.18	46.6		0.402	40.2	8.8
Cytosine..................	5.8	2.19	14.2		0.205	20.5	4.5
Uracil...	1.3	0.32	2.1		0.046	4.6	1.0,
Total nucleic acid..... ..			82.1	4.0		81.9	

* The nucleic acid preparation contained N 15.4, P 7.9 per cent.

far the major component of this nucleic acid. The molar guanine to adenine ratio was 2.4. It might be mentioned that in the analysis of a second less pure preparation of a pancreas nucleic acid which is not discussed in detail here, since it contained some protein and about 6 per cent of desoxypentose nucleic acid, the following figures were found: adenine 4.1, guanine 16.5 per cent.

The proportions and balances, computed as for yeast ribonucleic acid, are tabulated in Table VIII.

Sugar Component of Pentose Nucleic Acids

Preliminary experiments were carried out on the nature of the pentose liberated by the cleavage of the purine nucleotide moiety of the nucleic acids studied. It was of interest to ascertain the possibility of examining the sugars in minute amounts of hydrolysates by means of filter paper chromatography (20). This proved feasible, particularly when the development method for sugars recently described from this laboratory (21) was employed.

The paper used was Schleicher and Schüll, No. 597. The solvent system consisted of a mixture of 4 volumes of n-butanol, 1 volume of ethanol, and 5 volumes of water; the upper organic layer was used for the chromatographic separation which was permitted to proceed for about 12 hours at room temperature. In all experiments, adjacent chromatograms were made with authentic D-ribose and also with D-xylose which is the pentose nearest to ribose in partition behavior.

3 mg. portions of the yeast and pancreas nucleic acids each were hydrolyzed with 0.3 cc. of N H_2SO_4 at 100° for 1 hour. During this time the volume was kept approximately constant by the addition of a few drops of water. For the chromatograms 0.01 cc. portions of the hydrolysates were employed; the drops were neutralized on the paper by exposure to gaseous NH_3. Following the separation the paper strips were treated with m-phenylenediamine dihydrochloride as described before (21). The strongly fluorescent spots indicated the presence of one sugar only, which was in all probability D-*ribose*. The R_F values of the sugar components of both yeast and pancreas nucleic acids were 0.30 and coincided completely with the position of authentic ribose. D-Xylose, on the other hand, occupied an unmistakably different position, with an R_F value of 0.24.

Appendix: Characterization of All Nitrogenous Constituents in One Nucleic Acid Sample

In cases in which the scarcity of the available material precludes two separate weighings for the determination of the purines and the pyrimidines respectively, the following procedure may be employed, which involves the liberation of the purines by mild acid hydrolysis and the, at least partial, precipitation of the pyrimidine nucleotides by uranyl acetate in a manner similar to that followed with the purine nucleotides (compare (22)). With regard to the estimation of the purines the procedures are identical with the ones described above, and the figures found have the same significance; the figures for the pyrimidines, however, have at best a semiquantitative value, though the method may be of interest for the characterization of the pyrimidines in very small amounts of nucleic acid.

Between 15 and 25 mg. of the nucleic acid preparation, previously dried in a high vacuum at 60° for 3 hours, were weighed exactly into a small bomb tube, 1.5 cc. of N H_2SO_4 were added, and the sealed tube was heated for 1 hour at 100°. The hydrolysate was brought up to a volume of exactly 2.5 cc. with 0.1 N H_2SO_4, either directly or after adjustment to pH 0.8 to 1, as was described above in the section on the estimation of purines. A portion of this solution (0.3 cc.) served for the purine determinations.

An exact 2.0 cc. aliquot of the solution was placed in a centrifuge cup and adjusted to pH 7 with the help of a mixed indicator. Guanine, which precipitated in part, was centrifuged off and washed once with water. From the united supernatants the nucleotides were precipitated by the addition of a sufficient amount of a saturated (7 per cent) uranyl acetate solution. 10 minutes later the mixture was centrifuged and the supernatant brought to neutrality, at which time a second precipitation occurred. The supernatant was discarded and the united precipitates were dissolved in about 1 cc. of 2 N HCl in order to remove purine traces. The neutralization of this acidic solution brought about the reprecipitation of the uranium salts. The mixture was treated with 1 to 2 drops of the uranyl acetate solution, allowed to stand for 10 minutes, and then centrifuged. The sediment was dissolved or suspended in 0.3 cc. of about 100 per cent formic acid[5] and the solution transferred quantitatively to a bomb tube, two 0.1 cc. portions of formic acid being used for washing. The sealed bomb tube was heated to 175° for 2 hours; the cooled hydrolysis mixture was freed of a white sediment by centrifugation and the supernatant introduced into a 1 cc. volumetric flask. The sediment was washed as often as possible with small amounts of water which served to bring the volume to 1 cc. The chromatography and estimation of the pyrimidines then were carried out in the prescribed fashion.

The total of pyrimidines recovered in this manner was considerably lower than in the procedure discussed above for the quantitative estimation of pyrimidines. The loss was largely attributable to a disappearance of cytosine which in this case, however, was not accompanied by a rise in uracil. The following pyrimidine figures were, for instance, found for yeast ribonucleic acid: cytosine 4.3 and uracil 1.8 per cent, which corresponded to 64 and 78 per cent respectively of the authentic values given in Table V. For the pancreas nucleic acid the figures were cytosine 2.9 and uracil 1.0 per cent, amounting to a recovery of 53 per cent of the cytosine and of 83 per cent of the uracil reported in Table VII.

That this loss in cytosine could not be due to the partial solubility of the

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[5] While the uranium precipitate of cytidylic acid was completely soluble at this state, a small amount of an insoluble residue remained when hydrolysates of pentose nucleic acids were examined.

uranium salt of cytidylic acid itself was shown in a control experiment with cytidylic acid (Preparation 1). 99.8 mg. of the nucleotide were dissolved in 0.1 N HCl to give a volume of exactly 25 cc. It was found that the entire P contained in 1 cc. aliquots of this solution went into the precipitate produced by uranyl acetate. However, the uranium precipitates thus obtained, when heated at 175° with concentrated formic acid for varying periods, yielded cytosine values that were considerably lower than those given in Table II. The percentages of cytosine found were as follows: after 90 minutes heating 21.4, after 120 minutes 22.7, after 150 minutes 18.4.

DISCUSSION

Since this study is in many ways the first of its kind, the discussion may well begin with a brief consideration of its limitations and shortcomings. First of all, it deals solely with the *composition* of nucleic acids; its bearing on the problem of nucleic acid structure is only indirect. Although the recognition of structural principles requires an exact knowledge of the nature and the proportions of all constituents, the findings reported here demand no particular assumption with respect to a specific arrangement of the components or to the type of linkage holding them together. Secondly, it must be understood that all figures presented refer to the hydrolysates only. Groupings that are not cleaved in the course of hydrolysis and compounds that are destroyed during the cleavage of the nucleic acid or that are not liberated at all will, of course, escape detection. The hydrolysis may, on the other hand, lead to the production of artifacts, not present as such in the starting material. These reservations naturally apply to the constituent analysis of all complicated organic substances.

The quality of the nucleic acid preparations also may be responsible for a number of errors. It is known that pentose nucleoproteins in general require a much more drastic treatment for the detachment of the nucleic acid than is the case with the corresponding desoxypentose nucleic acid complexes; and the commercially available preparations of yeast ribonucleic acid probably are badly degraded, as indicated by the numerous purification procedures found in the literature (6) and also by the not infrequently discordant results obtained with different specimens (2). The situation is more favorable with respect to the pentose nucleic acid from pancreas and even more so as regards the desoxypentose nucleic acids which will be discussed in a forthcoming publication. In any event, general principles of composition can already be recognized in the pentose nucleic acids.

That satisfactory methods of hydrolysis are among the most important requisites for the complete characterization of the composition of nucleic acids requires no added emphasis. The liberation of the purines is generally

assumed to be achieved by mild acid hydrolysis (compare p. 223 in (1) and also (19)); and this was definitely proved in the present study with purified adenylic and guanylic acids from which adenine and guanine in the respective yields of 95 and 100 per cent of the expected amounts were isolated. The complete release of the pyrimidines presents a much more difficult problem. The method commonly employed, *viz.* prolonged autoclaving with strong mineral acid at a high temperature, must, in the light of the findings reported here, have led to very erroneous conclusions. It has now been found that under these conditions a large part of the cytosine of the nucleic acid is converted to uracil. It was, therefore, necessary to develop a hydrolysis procedure that would permit the attainment of the maximal liberation of constituents without the production of artifacts or of a shift in proportions. Concentrated formic acid was finally chosen for this purpose. This treatment yielded values that were often higher, but in no case lower, than those for total pyrimidine content found with strong mineral acid. Furthermore, since it effected no appreciable conversion of cytosine to uracil, the important question of the presence of uracil as such in the pentose nucleic acids became susceptible of an answer.

The great advantage of the estimation method employed here lies in the fact that it makes possible a complete survey of the distribution of purines and pyrimidines in minute amounts of nucleic acid hydrolysates[6] and that it is capable of distinguishing between the different nitrogenous constituents. Since the total hydrolysate is subjected to a partition between solvents, all components, regardless of their physical properties, have an equal chance of being demonstrated without having to undergo wasteful conversions into derivatives.

The accuracy of the chromatographic separation of the purines and pyrimidines has been discussed in the preceding communication (7). It now remains to consider the faults that could vitiate the analytical results on nucleic acids. If pyrimidines had partly been liberated in the course of the mild hydrolysis employed for the release of purines, slightly too high a value could have resulted for adenine, due to contamination with uracil and, perhaps, also with cytosine. This is, however, unlikely, not only because of the spectroscopic controls that were invariably applied, but also because of the well known stability of the pyrimidine nucleotides. The latter property could, however, be a cause of error in the pyrimidine determinations; this will be taken up later. Another error could have been

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[6] Even smaller quantities of nucleic acid than were used here could doubtless be employed for the estimations, if the hydrolysates were adjusted to a lesser volume, or if, without volume adjustment, a known amount of a substance were added to the hydrolysates which, serving as a marker, could be separated and estimated quantitatively on the chromatogram.

introduced by the contamination of the pyrimidine fraction with purines that had not been removed completely as the hydrochlorides. This is even less likely; in the chromatographic procedure used, the cytosine fraction could have contained traces of hypoxanthine which, however, probably is not a common nucleic acid constituent and is quite different spectroscopically from cytosine. The absorption maxima of uracil and of adenine, its possible contaminant, are nearer, but since no uracil spot was detected in the corresponding hydrolysates of desoxypentose nucleic acids, contamination with adenine is not likely to have played an important rôle.

Before the results presented in this study are compared with those of previous workers, one additional point should be stressed. The inspection of Tables VI and VIII will show that in both nucleic acid preparations a certain proportion (15 to 18 per cent) was not accounted for. Several reasons could be responsible for this deficit. It could, for instance, have resulted from the summation of hydrolysis losses affecting equally the estimations of all the purines and pyrimidines. One observation would seem to favor this assumption. When the total number of gm. atoms of nitrogen found in the hydrolysates is divided by the total number of moles of the four bases in which they are contained (see molar proportions in Tables VI and VIII), the quotients, which indicate the average nitrogen content of each nitrogenous constituent, are in very good agreement with the atomic nitrogen to phosphorus ratio calculated from the analytical values found for the intact nucleic acids. For yeast ribonucleic acid this quotient is 4.1, the atomic N:P ratio 4.2; the corresponding figures for the pancreas nucleic acid are 4.3 and 4.3. This agreement suggests that the hydrolyses did not result in a considerable fractionation.

Another possibility is that the nucleic acids contained small amounts of unidentified components which either were resistant to hydrolysis or gave rise to substances that could not be demonstrated by the chromatographic methods. In this connection, attention may be drawn to the behavior of isolated cytidylic acid toward hydrolysis, discussed above in conjunction with the experimental findings. Several peculiarities exhibited by ribonucleic acids, e.g. the lability of the internucleotide linkages toward alkali, cannot be explained on the basis of the currently assigned structures; and these problems will have to be investigated further in connection with the behavior of isolated pyrimidine nucleosides and nucleotides. It will be remembered that dephosphorylation of the nucleic acids appeared complete under the conditions of hydrolysis (Table II); but it is not impossible that a small proportion of differently linked pyrimidines was not liberated by acid hydrolysis. Chromatographic separation studies on the distribution of the constituent nucleotides and other investigations, which are being carried out at present, will perhaps contribute to a decision.

The identification of the sugar component that is associated with the purine moiety of the pentose nucleic acids studied here was attempted, in order to test the possibility of applying a chromatographic microprocedure to the investigation of the carbohydrates present in minute amounts of nucleic acids. Only one sugar was detected in the hydrolysates of the yeast and the pancreas nucleic acids. It was identical in partition behavior with D-ribose, which is in accordance with the original findings of Levene and Jacobs (23).

When the tetranucleotide structure of yeast ribonucleic acid is discussed in the literature (compare, for instance, (3) p. 198), reference is usually made to a publication of Levene (24) that is said to provide the evidence for the occurrence in this nucleic acid of the two purines and the two pyrimidines in equimolecular proportions. Actually, this is far from correct. From 10 gm. of nucleic acid (N 15.2, P 8.6 per cent) 2.0 gm. of adenine picrate and 1.0 gm. of guanine were isolated. The same quantity of nucleic acid yielded cytosine, as what is described as 3.0 gm. of a crude picrate, and uracil whose isolation was reported without any indication of its weight. The nitrogenous bases were, therefore, distributed as follows: adenine 7.1 per cent (0.19 mole per mole of P); guanine, in good agreement with the present findings, 10 per cent (0.24 mole); cytosine (if the crude derivative is considered as cytosine picrate) 9.8 per cent (0.32 mole); no value can be assigned to uracil. The corresponding molar proportions, *viz.* adenine 1.0, guanine 1.3, cytosine (?) 1.7, hardly lend themselves to the formulation of a regular tetranucleotide. The figures assembled in Table VI in the present paper provide, in fact, much better evidence of regularity, but further considerations of this kind should be postponed.

Most of the other evidence is of a more circumstantial nature, *e.g.* the rates of liberation of phosphoric acid (25, 26) and of sugar (26, 27), the calorimetric behavior of yeast nucleic acid (28), the amount of total purine nitrogen liberated (19), etc. A discussion of the analytical data submitted in a preliminary form by Loring *et al.* (29) should, in view of a recent note (30), await the presentation of experimental details.

The pentose nucleic acid of pancreas, while much investigated, has not had as important a part as yeast nucleic acid in the development of the conception of nucleic acid structure, though its peculiar composition early served to draw attention to the far from simple problems involved. It was suspected of not fitting into the pattern of a regular tetranucleotide and was in turn formulated as a guanylic acid complex of ribonucleic acid and as a hexa- or pentanucleotide. Hammarsten (31) assumed a guanine to adenine ratio of 3:1, Steudel (32) of 4:1, Levene and Jorpes (12) found in different preparations 3.3 and 4.6 times as much guanine as adenine, and Jorpes (11) twice as much guanine as adenine. Since some of the guanylic acid present in this material appears to be bound rather feebly, it is probable

that different methods of isolation led to products of somewhat varying composition, quite apart from the uncertainties inherent in the estimation methods.

It will be seen that the findings summarized in Table VIII correspond to about 4 molecules of adenine and 5 of cytosine per 10 molecules of guanine. The ratio of purine to pyrimidine nitrogen was very high. Since pancreas is rich in ribonuclease which appears to act preferentially on the pyrimidine nucleotide portion of ribonucleic acids (33, 34), it is not impossible that some pyrimidine was liberated in the course of the preparation of the material.

One more point remains to be considered, namely the presence of uracil in the pentose nucleic acids. That cytosine is converted to uracil with relative ease has long been known (35); and in view of the extremely energetic methods commonly used for hydrolysis it is natural that the possibility of uracil being an artifact has formed the subject of lively controversies. Kowalevsky (36), in an investigation of yeast ribonucleic acid carried out in Steudel's laboratory, attempted to demonstrate the exclusive presence of three nitrogenous bases which she claimed to occur in the following molar proportions: adenine 1, guanine 1.6, cytosine 1.1. While the concentration of uracil in the hydrolysates of both pentose nucleic acids examined in the present study is very low, there is every reason to believe that it is a genuine constituent of the preparations. To what extent, however, the effect of deaminases and of other enzymes acting on the nucleic acids during their isolation may contribute to their final composition is a problem that will have to be considered separately.

SUMMARY

The methods for the separation and estimation of adenine, guanine, cytosine, and uracil in minute amounts, described in the preceding communication, were applied to a study of the distribution of these nitrogenous constituents in hydrolysates of the ribonucleic acids of yeast and of pancreas. Since it was shown that under the customary conditions of hydrolysis with strong mineral acid cytosine was largely converted to uracil, concentrated formic acid was chosen as the hydrolyzing agent.

The presentation of detailed methods for the preparation of the hydrolysates and the estimation of the individual components is followed by a consideration of the proportions in which the purines and pyrimidines were found in the hydrolysates. A procedure, permitting the characterization of the nitrogenous constituents in very small quantities of nucleic acid,

and the application of chromatography on filter paper to the identification of the carbohydrate components of nucleic acids likewise are described.

The paper, which includes studies of the hydrolysis behavior of isolated nucleotides, concludes with a critical discussion of the findings.

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A NEW PREPARATION OF CRYSTALLINE ANTERIOR PITUITARY GROWTH HORMONE*

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WITH THE TECHNICAL ASSISTANCE OF MARJORIE CAPPIELLO

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The isolation and identification of the anterior pituitary growth hormone as a pure protein were first achieved by Li, Evans, and Simpson (1, 2) in 1944–45. Their method is a beautiful example of the classical procedures of protein fractionation by the controlled use of salt and of hydrogen ion concentrations. It owes its distinction to the proof that the growth-promoting activity of anterior pituitary extracts, first demonstrated by Evans and Long (3), is attributable to an individual protein. The disadvantages of the method lie in the somewhat low extraction efficiency from acetone powders of the anterior pituitary glands, in the tedium of the many repeated steps of the process, in the time consumed, in the losses inevitably associated with repeated manipulations, and in the resulting low yields of product (of the order of 60 mg. per kilo of fresh anterior pituitary glands). The method attains its objective of isolating pure growth hormone, and it secures information about the properties of the protein that is essential in the development of an improved method, but is impractical for obtaining the hormone in the quantities required for extensive experimental and clinical investigation.

It has been found that a fractionation of calcium hydroxide extracts of fresh bovine anterior pituitary glands, by means of ethanol at low temperatures along the lines successfully exploited by Cohn and his colleagues (4) in the separation of the plasma proteins, yields an abundance of crude fractions with high growth-promoting activity. Re-solution of these active fractions in dilute potassium chloride, followed by removal of the bulk of impurities in a precipitate formed at pH 5.0 and by a fractional precipitation with ethanol, starting at pH 8.5 to 8.7, yields a crystalline protein, electrophoretically homogeneous, which from its biological activity and other properties is identified as the anterior pituitary growth hormone. The yields of the crude primary fractions average about 33 gm. per kilo of fresh glands. The yields of the purification procedure lie between 8 and 16 per cent. On this basis, the new method can be expected to produce quantities

of pure or nearly pure crystalline growth hormone of the order of 3 gm. per kilo of fresh glands.

Methods

The growth-promoting action of the various fractions was measured by their effects on the body weight of male hypophysectomized rats,[1] of about 100 gm. body weight, brought about by the daily intraperitoneal injection of solutions of the test material in normal saline for 10 days. In most instances the effects on growth were checked by measurements of the width of the proximal epiphyseal cartilage of the tibia, as described by Evans, Simpson, Marx, and Kibrick (5). Adrenocorticotropic activity was measured by the method of Sayers, Sayers, and Woodbury (6). Glycostatic activity was measured by the effects of two intraperitoneal injections of the test substance in normal saline solution on the level of the muscle glycogen of 200 to 250 gm. hypophysectomized male rats fasted for 24 hours (7). All of the experimental animals were maintained on a diet of laboratory chow in a room at a constant temperature of 25.5°.

Determinations of pH were made with the glass electrode.

Method of Isolation—The anterior lobes were carefully dissected from frozen bovine pituitary glands and were stored in a freezer until required. Small lots of 300 to 500 gm. were used for the individual runs.

The glands are prepared for extraction by thawing them slightly, mixing the separated glands with coarsely ground solid carbon dioxide, and grinding them to a fine powder by two or three passes, with successively closer plate settings, through a Straub dry food grinder previously chilled with solid carbon dioxide. The carbon dioxide is allowed to evaporate, leaving a cold pink mush of the ground glands.

All subsequent operations are carried out in a cold room at 0–5°.

The ground glands, free of carbon dioxide, are suspended in dilute calcium hydroxide solution, pH 11.5, and stirred mechanically for 24 hours. 2 liters of calcium hydroxide solution are used for 300 to 350 gm. of fresh frozen glands. During the early hours of stirring, the pH of the mixture must be maintained at 11.5 by occasional additions of solid calcium hydroxide. The pH becomes steady after 3 or 4 hours, and, in the conditions in our cold room, it is usually not less than pH 11.0 at the end of 24 hours stirring.

The pH of the mixture is adjusted to 8.5 to 8.7 by bubbling in carbon dioxide gas, care being taken not to overrun, and the mixture is allowed to settle overnight. It is thereafter centrifuged, the opalescent pink supernatant solution is decanted, and the residue is discarded.

[1] The hypophysectomized rats used in the growth tests were purchased from Hormone Assay, Inc., of Chicago, Illinois. The condition, survival, and uniformity of response of these animals were excellent.

To the supernatant solution, rapidly stirred, a calculated volume of a mixture of equal parts of 95 per cent ethanol and water (1:1 ethanol) is added dropwise, at a rate of about 60 ml. per hour, until the concentration of ethanol in the mixture is 12 per cent. The precipitate that forms is centrifuged off, Fraction A.

To the 12 per cent ethanol supernatant solution, vigorously stirred, 1:1 ethanol is added dropwise until the ethanol concentration is 24 per cent. The precipitate is centrifuged off, Fraction B.

The 24 per cent ethanol supernatant solution is adjusted to pH 6.8 with 4 N hydrochloric acid, and the resulting precipitate centrifuged off, Fraction C.

The supernatant solution is adjusted to pH 4.6 with 4 N hydrochloric acid, and the resulting precipitate centrifuged off, Fraction D.

To the 24 per cent ethanol supernatant solution, pH 4.6, vigorously stirred, 95 per cent ethanol is added dropwise until the ethanol concentration of the mixture is 40 per cent. The resulting precipitate is centrifuged off, Fraction E.

The clear pink supernatant solution is discarded.

Fractions A to E are each suspended in distilled water and lyophilized. They constitute the crude primary fractions. Of these, Fractions A, B, and C contain nearly all of the growth activity, and these fractions may be purified in the following steps.

A 0.5 per cent solution of Fraction A, B, or C in 0.1 N potassium chloride solution is made with the aid of 1 N potassium hydroxide, added until the pH of the mixture is 11.0. Solution is usually complete; if it is not, the solution is cleared by centrifuging.

The pH of the solution is adjusted to 5.0 with 4 N hydrochloric acid. The resulting precipitate is centrifuged off, suspended in one-half the original volume of 0.1 N potassium chloride solution, and saved.

The supernatant solution, water-clear and usually nearly colorless, is adjusted to pH 8.5 to 8.7 with 1 N potassium hydroxide, and a calculated volume of 1:1 ethanol is added dropwise (rate, 30 ml. per hour) to the rapidly stirred solution until the ethanol concentration of the mixture is 5 per cent. The resulting precipitate, which exhibits some crystals on microscopic examination, is centrifuged off and saved.

To the water-clear 5 per cent ethanol supernatant solution, vigorously stirred, the calculated volume of 1:1 ethanol is added dropwise until the ethanol concentration of the mixture is 20 per cent. A crystalline precipitate forms slowly and steadily throughout the period of addition, and the process appears to be complete by the end of the addition. The crystalline precipitate is pure, or nearly pure growth hormone. It is collected by centrifugation, suspended in distilled water, dialyzed against distilled water

until salt-free, and lyophilized. A photograph of the crystalline product is presented in Fig. 1.

The precipitate, pH 5.0, together with the 5 per cent ethanol precipitate, may be redissolved in one-half the original volume of 0.1 N potassium
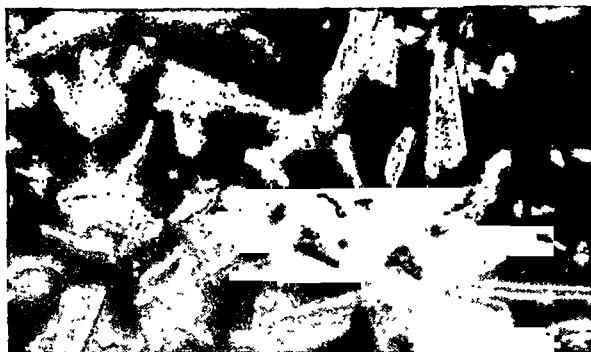
FIG. 1. Growth hormone crystals, photographed in their mother liquor. Dark-field; magnification × 430.

TABLE I

Yields of Total Solids and of Crude Fractions A to E in Nine Extractions of Fresh Beef Anterior Pituitary Glands

Experiment No.	Glands, fresh weight	Solids in extract		Fractions					
				A	B	C	D	E	A + B + C
	gm.	gm.	gm. per kg.	gm.	gm.	gm.	gm.	gm.	gm. per kg.
12	304			0.6	1.9	4.0	1.2	0.4	21.7
46	304	23.5	77.3	4.6	2.3	4.6	3.5	1.2	38.3
62	305	24.9	81.6	16.6*			1.4		55.3
82	335	32.1	95.8	9.5	3.4	2.3	2.6	0.9	45.2
115	350	23.8	68.0	4.5	8.6	2.8	1.9	0.6	45.3
143	350	14.6	41.7	2 9	1.3	0.4	1.1		13.1
168	500	28.1	56.2	10.3	1.9	0.2	1.9		24.8
194	430	26.4	61.4	5.8	2.0	0.6	2.0		19.6
218	382	23.5	61.5	8.7*			0.3		22.7

* Fractions A, B, and C not separated.

chloride solution with the aid of 1 N potassium hydroxide and put through the procedure once more to yield additional crystalline product. The products obtained in a second and third rework of the precipitate at pH 5.0 are in small yield and are not homogeneous.

Yields

A summary of the yields of total solids in the crude extracts of several separate runs and of the amounts of Fractions A to E is presented in Table I. The total solids of the pH 8.5 to 8.7 extract, measured by lyophilizing

an aliquot of the extract, average nearly 69 gm. per kilo of fresh glands. At an average of 12 per cent of nitrogen, this amounts to 8.38 gm. of nitrogen per kilo of fresh glands. Li, Evans, and Simpson (2) report a yield of 4.37 gm. of nitrogen per kilo of fresh glands in one whole extract of an acetone powder. It seems probable that active material may be more readily and completely extracted from the finely ground fresh glands than from the acetone powder.

The yields of Fractions A to E vary somewhat from run to run, and the distribution of material, especially between Fractions A, B, and C, is also irregular. This may be due in part to differences between different lots of glands, and in part to the effects of evaporation from open vessels, during vigorous stirring in a room in which a strong flow of cold air is maintained, on the final ethanol concentration attained at each step. There is also a

TABLE II

Summary of Yields of Crystalline Growth Hormone from Experiment 194

	gm	gm
Fresh glands extracted... . ..		430
Fraction A...... . .		5 8
Crystals (1st run). ..	0 939	
" (2nd ").....	0 177	
Fractions B + C....... .		2 5
Crystals (1st run).	0 159	
" (2nd ")	0.072	
Total crystals........	1 347	

Yield, 2.99 gm. of crystals per kilo of fresh glands.

slow drift of pH from its starting point of 8.5 to 8.7 to about 7.5 to 7.7 at the end of the second ethanol addition, and this may influence the distribution of material between Fractions A and B. More consistent yields of material in each fraction might be attained by carrying out the procedure in closed vessels. The combined yield of Fractions A, B, and C is about 33 gm. per kilo of fresh glands. Slightly less than one-half of the total solids in the extract is collected in the five crude fractions. The final supernatant solution has not yet been explored for its content of other kinds of pituitary hormone activity.

The yields of crystalline material obtained from Fractions A, B, and C of one run are outlined in Table II. In this instance, the yield is about 17 per cent of the crude starting material. This step of the procedure was developed during attempts to purify the crude crystalline preparations described in a preliminary report of the method (8). In these trial procedures, yields of electrophoretically homogeneous material of the order of 8 to 20 per cent of the starting material were obtained. It may therefore n

unreasonable to expect yields of crystalline material by the new and simpler procedure to be of the order of 3 gm. per kilo of fresh anterior pituitary glands.

Electrophoretic Analysis

Because of the uncertainties of the growth assay and the fact that Fractions A, B, and C already exhibit an order of growth activity such that it is
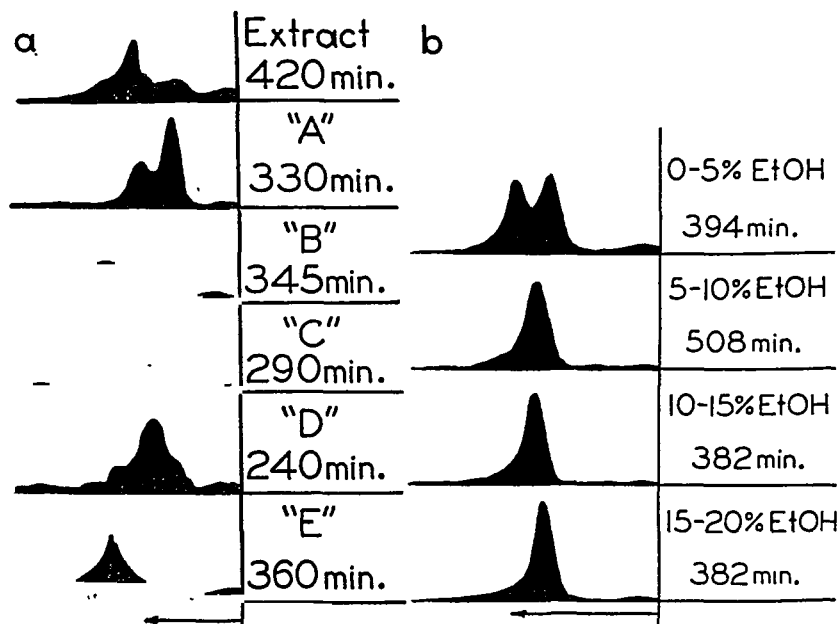
FIG. 2. (a) Tracings of the electrophoretic patterns of the crude extract and of the five primary fractions. The large slow peaks in Fractions A and B are identifiable by their mobilities with the growth hormone. (b) Tracings of the electrophoretic patterns of successive fractions obtained during the fractional crystallization of the growth hormone from KCl solution with ethanol. All of the tracings are of ascending boundaries. Glycine buffer pH 10.0, ionic strength 0.17, temperature 4°, current 17 milliamperes.

difficult to discriminate them biologically from more highly purified materials, the progress of purification has been followed electrophoretically. The Tiselius apparatus employed is equipped with the Longsworth modification of the Philpot scanning device. For many of the routine observations a buffer of the composition, glycine 0.1 M, sodium hydroxide 0.67 M, sodium chloride 0.1 M, pH 10.0, ionic strength 0.17, is employed, since it was found that good clear solutions of 1 per cent protein concentration were most easily obtained in this buffer, especially with the cruder fractions. In Fig. 2, a are seen the electrophoretic patterns in glycine buffer pH 10 of the

whole extract and of the five crude fractions. The large slow peak seen in Fractions A, B, and C has a mobility characteristic of that of the growth hormone. The corresponding peaks in Fractions D and E comprise a relatively small proportion of the areas of the patterns. This is in good accord with the biological data summarized below. The different patterns of Fractions A, B, and C, taken together with the fact that a colored contaminant of the fractions increases in amount from Fraction A to C, seem to justify taking these fractions separately, as described. In one run these fractions were combined by making a straightforward addition of 1:1 ethanol to a concentration of 24 per cent of ethanol. Since the final purification steps had not been developed at that time, it cannot be said whether an "ABC" fraction would be as easily susceptible of purification as the separate fractions are. This point will be examined, since a considerable economy of effort can be made if the first three fractions can be taken as one.

The advantage of the final fractional precipitation with ethanol is illustrated in Fig. 2, b, which is taken from an experiment in which separate precipitates were collected at 5, 10, 15, and 20 per cent of ethanol. Most of the impurity carried over from the precipitation at pH 5.0 appears to be removed at the lowest concentration of ethanol. There is no significant difference between the other three fractions, which are uniformly crystalline. They are therefore taken as one fraction in the routine version of the method.

The electrophoretic patterns of a crystalline preparation of growth hormone in glycine buffer at pH 10 and in acetate buffer at pH 4 are illustrated in Fig. 3. The material appears to be homogeneous, and the mobilities observed at each hydrogen ion concentration correspond closely with the mobilities observed by Li, Evans, and Simpson (2) and by Li[2] for their preparation of pure growth hormone.

This same preparation is being examined for us in the ultracentrifuge by Dr. Emil L. Smith of the University of Utah School of Medicine. In glycine buffer at pH 9.4 and at protein concentrations of 1 and 0.5 per cent, only a single sedimenting boundary is observed, and the rate of sedimentation is of an order indicating a molecular weight of about 49,000 for the material. A more complete ultracentrifugal analysis will be reported at another time, but this preliminary information affords additional evidence of the purity of the crystalline product and of its identity with the pure growth hormone isolated by Li, Evans, and Simpson (2).

Biological Activity

The observed effects of the crude Fractions A to E upon growth and upon the width of the proximal epiphyseal cartilage of the tibia in hypophysecto-

[2] Li, C. H., personal communication.

mized male rats are summarized in Table III.   It will be seen that Fractions A, B, and C bring about growth increments of 2 gm. or more per day at a dose level of 100 γ per day.   Since the relationship between dose and
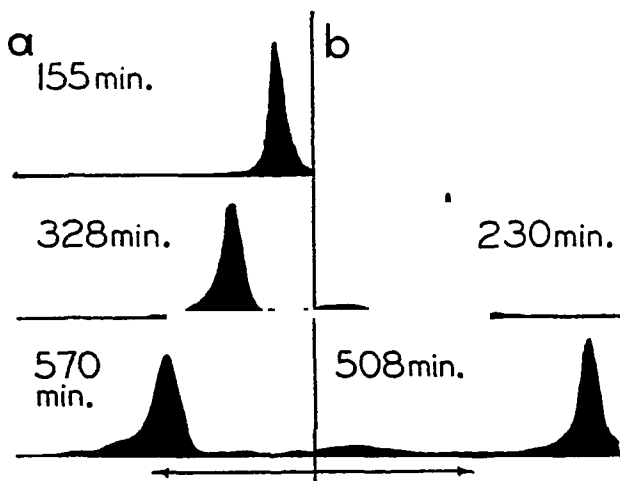
FIG. 3. Tracings of the ascending boundaries observed during the electrophoresis of crystalline growth hormone.   (a) Glycine buffer pH 10.0, ionic strength 0.17. (b) Acetate buffer pH 4.1, ionic strength 0.13.   Temperature 4°, current 17 milliamperes.

TABLE III

Effect of Crude Primary Fractions upon Growth and Width of Tibial Epiphyseal Cartilage in Hypophysectomized Rats

Fraction No.	Daily dose	10 day increase in	
		Body weight	Epiphyseal width
	mg.	*gm.*	*μ*
194-A	0.10	28.3	204
143-B	0.01	8.3	102
12-B	0.10	23.0	
12-C	0.10	21.0	
168-D	0.10	7.6	55
115-D	1.00	18.3	198
115-E	1.00	4.6	75

Each figure is the mean of three or four rats.

response is a logarithmic one, these fractions already exhibit unit activity; that is, they can be expected to bring about growth increments of 1 gm. per day at dose levels of 10 γ per day.   A full dress assay in an elaborate experimental design is therefore necessary to distinguish the growth potency of these three fractions from that of the crystalline hormone.   Fractions D

and E are much less active, which accords with the electrophoretic evidence that an element identifiable with the growth hormone forms only a minor part of the total electrophoretic pattern. In general, the effects of the different fractions in the different doses upon the widths of the tibial epiphyses correspond very well with their relative effects upon growth.

The growth-promoting effects of the crystalline hormone are summarized in Table IV. Fractions 198-A, 198-B, and 198-C were obtained with 0 to 5, 5 to 10, and 10 to 15 per cent of ethanol respectively. The first of these fractions, as one might expect from the electrophoretic patterns of Fig. 2, *b*, appears to be less active than the others. At the dose level of 20 γ daily, the crystalline fractions exhibit both growth and cartilage effects comparable to those observed with the pure growth hormone of Li, Evans, and Simpson (2).

TABLE IV

Effect of Purified Growth Hormone on Growth and Width of Tibial Epiphyseal Cartilage in Hypophysectomized Rats

Fraction No.	Daily dose	10 day increase in	
		Body weight	Epiphyseal width
	mg.	*gm.*	*μ*
198-A	0.02	12.3	119
198-B	0.02	16.6	159
198-C	0.02	14.3	150
202-3-BCD	0.02	15.8	180
	0.10	25.0	207

Each figure is the mean of three or four rats.

The crystalline material has been tested for adrenocorticotropic activity by the method of Sayers, Sayers, and Woodbury (6). A dose of 0.5 mg. per 100 gm. of body weight, injected intravenously into each of four 1 day-hypophysectomized rats immediately after the removal of one adrenal gland, produced in 1 hour an average fall in the concentration of ascorbic acid of only 23 mg. per cent in the remaining adrenal gland as compared with that of the control gland. This corresponds to the effect of 0.2 to 0.3 γ of purified adrenocorticotropic hormone, a negligible contamination of the order of 4 to 6 parts in 10,000.

Complete information is not yet available on the other kinds of pituitary hormone activity present in the crystalline growth hormone. A detailed assay of a cruder crystalline preparation made by the old procedure (8), which showed two components electrophoretically, yielded the following results:[3] (*a*) adrenocorticotropic hormone, less than 1.25 per cent of Armour

LA1A standard; (b) prolactin, less than 0.1 per cent of Armour standard (16 international units per mg.); (c) gonadotropic, negligible effects on uterine and ovarian weights of hypophysectomized female rats in 4 days at dose levels of 0.4 and 0.8 mg. per day; (d) thyrotropic hormone, 7.2 per cent of Armour standard or about 0.1 Evans chick unit per mg.; (e) pressor activity, less than 0.01 U. S. P. reference posterior pituitary units per mg.; (f) oxytocic activity, less than 0.004 U. S. P. reference posterior pituitary units per mg. This crude material is therefore relatively free of other kinds of anterior and posterior pituitary hormone activities, and it is to be expected that the crystalline hormone, which satisfied at least three of the criteria for purity, will prove to be cleaner in these respects.

. All of the active growth hormone preparations examined so far, including the purest crystalline preparations, have exhibited excellent glycostatic activity, which has become more intense with increasing degree of purification. A detailed account of the experiments will be given elsewhere, but it may be said, on the basis of the present observations, that the activity of the anterior pituitary gland in maintaining normal levels of muscle glycogen in the 24 hour fasted hypophysectomized rat, first observed by Russell (7), appears to be a property of the growth hormone.

Work is in progress on a detailed chemical and physicochemical study of the crystalline growth hormone prepared by the present method, in order to compare the properties of this material with those described by Li *et al.* (2, 9, 10) for their pure growth hormone.

Attention has so far been concentrated on developing the new method for isolating growth hormone. It is hoped, however, that the principles and procedure of the new method may be applied to the efficient isolation of other hormones of the anterior pituitary gland.

SUMMARY

A new method is described for the preparation of crystalline growth hormone from fresh bovine anterior pituitary glands. The method depends upon the fractionation with ethanol at low temperatures of a calcium hydroxide extract of the ground, fresh glands. The crude fractions obtained at this stage are purified by an isoelectric precipitation of contaminating material from dilute salt solution, followed by a fractional crystallization with ethanol. The yields of the crystalline product, which is electrophoretically and ultracentrifugally homogeneous, are of the order of 3 gm. per kilo of fresh glands. The crystals have the expected effects upon body growth and upon the width of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats. They are also the most active glycostatic hormone preparations yet isolated; so that it appears that glycostatic activity is one of the properties of the growth hormone.

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NOTES ON MYOGLOBIN PREPARATION AND IRON CONTENT

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Since the first description by Theorell (11) of a process of isolating and purifying myoglobin from horse heart by crystallization, several workers have succeeded in preparing myoglobin by Theorell's procedure. Roche and Vieil (8) and Rossi and Aragona (9) have described methods less arduous than Theorell's. Their methods, however, sacrifice yield to gain simplicity.

We have developed a modification of Theorell's procedure for preparing horse heart myoglobin in which the more than twenty steps described by him are reduced to about ten with no sacrifice of yield. During the work, difficulty was experienced in attaining purity of the preparations based on the value for the iron content, 0.345 per cent, reported by Theorell (11) and others. It became apparent that the value 0.345 per cent iron is greater than the value computed from the ratio in myoglobin of iron content (1 atom per molecule) and the molecular weight. The results of analyses of our preparations indicate that the value computed from the molecular weight derived from sedimentation and diffusion constants can be used as a criterion of purity of myoglobin.

Preparation

At a slaughter-house, horse hearts are removed as soon as possible and perfused through the coronary arteries with tepid 0.85 per cent NaCl solution (made on the site with warm tap water) until the solution emerging from the veins is colorless. About 15 liters of solution are used per heart. The auricles and as much fat and connective tissue as practical are trimmed away from the ventricles. In the laboratory the work is carried out at 1–5° as far as possible. The ventricular flesh is ground by an electrically driven meat chopper, and the ground meat is mixed with a chilled volume of water equal in ml. to the weight of the meat in gm. The mixture is stirred well and left in the cold overnight.

The following morning the extract is separated by centrifuging at 1000*g* or more. The extract is then partially purified by making the solution 3 M (50 per cent of saturation) with ammonium sulfate and separating the precipitate thus salted out by either centrifugation (1000*g*) or filtration. The supernatant solution is put into cellophane sausage bags 3 or 4 inches

in diameter, which are then immersed in a chilled bath of saturated ammonium sulfate that contains an excess of the salt and is adjusted to pH 7.1 to 7.3 with NH_4OH. The solution outside the bag is stirred with an electric stirrer for 24 hours.

Crystals of myoglobin appear in a day or so gathered in typical clusters (11), the form of which resembles sheaves of grain. The clusters increase in size for 2 or 3 weeks and reach lengths up to 1 ml. The myoglobin can be left in the baths indefinitely at about 5°. (We have stored it thus for 1 year.) To isolate the crystals from the amorphous impurities, the contents of the dialysis bag are centrifuged at $1000g$ for 10 minutes. The supernatant solution with as much of the amorphous material as possible is aspirated from the layer of crystals. The remaining precipitate of crystals and amorphous matter is resuspended in saturated ammonium sulfate[1] and centrifuged at $200g$ to $300g$ for 5 minutes. The supernatant solution is again removed by aspiration. The process of resuspension, centrifugation, and aspiration is repeated three times. The precipitate of crystals is next suspended in saturated ammonium sulfate[1] in a 500 ml. graduated cylinder. After 24 hours the larger crystals have settled to the bottom and the solution above them is removed by aspiration. This process of suspension, settling, and aspiration in the cylinder is carried out six times.

After the final washing, the ammonium sulfate is diluted until the myoglobin is dissolved. The solution is then dialyzed in a cellophane bag, 0.75 to 1.0 inch in diameter, in the cold against running distilled water for 24 hours or until a portion of the extract gives no indication of sulfate when tested with 10 per cent barium chloride. The dialysis is carried out on a rocker arm with a bubble in the bag to assure mixing. When the solution is removed from the bag, it contains a light precipitate which is apparently of no consequence and is removed by filtration. The solution of myoglobin thus obtained can be stored indefinitely in a refrigerator if a drop of chloroform is added.

Iron Content

In his original publication, Theorell (11) presented four determinations of iron in horse heart myoglobin which averaged 0.345 per cent. Later, Theorell and de Duve (12) obtained 0.340 per cent in human myoglobin. Either of these values is sometimes claimed to be the iron content of hemoglobin (4, 5, 11), and since myoglobin, like hemoglobin, contains 1 iron atom per unit of heme, it has been accepted as the iron content of myoglobin. Millikan (5) gives 0.345 per cent in a review of research on myo-

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[1] In order to lessen the possibility of contaminating the myoglobin with iron contained as an impurity in ammonium sulfate, the saturated solution was filtered after standing several weeks and the iron in it had precipitated.

globin, and Rossi and Aragona (9) state that they obtained this proportion repeatedly. Drabkin (4) found 0.340 ± 0.002 per cent. When used to calculate the molecular weight of myoglobin, 0.345 per cent iron gives 16,186. The value 0.340 gives 16,424.

The molecular weight of horse heart myoglobin has been determined by five methods. These are listed in Table I, together with the calculated values for iron content on the assumption of 1 atom of iron per molecule. It is to be noted that the results are consistently lower than that obtained by Theorell. Zinoffsky (14) found, by painstaking methods, that the iron content of horse hemoglobin is also less than 0.345 per cent; he obtained 0.335 per cent. Valer (13), working with care equal to Zinoffsky's, obtained 0.330 per cent.

TABLE I

Molecular Weight of Myoglobin from Literature and Calculated Iron Content for Each Observation

Mol. wt.	Iron calculated from mol. wt.	Method of determination of mol. wt.	Observer and reference No.
	per cent		
16,850	0.331	Osmotic pressure	Roche and Vieil (8)
17,200	0.325	Sedimentation and diffusion	Polson (6)
17,500	0.319	Sedimentation-equilibrium	" (10)
17,534*	0.318	Bergmann and Niemann (1)	Roche and Derrien (7)
17,600	0.317	Rule of simple multiples	Pedersen (10)

* Roche and Derrien actually give 16,934, but they neglected to add the value of the molecular weight of 1 heme molecule (600) to the weight of the globin portion determined by them.

The molecular weight 17,500 (Table I), according to Pedersen (10), is the mean of four values obtained from determinations of the sedimentation equilibrium by Polson. Polson (6) also determined the molecular weight of myoglobin from the same preparation from sedimentation and diffusion constants, with correction for particle size. In this instance he obtained 17,200. Technical difficulties prevent the exact measurement of these values by sedimentation and diffusion methods and the publications cited give few of the data necessary for statistical evaluation. Polson (6) presents five experimental diffusion constants taken on two concentrations of myoglobin, which vary less than 1.1 per cent from the mean; however, according to Edsall (3), Svedberg has estimated the probable error in diffusion and sedimentation constants as 2 to 3 per cent and in calculated molecular weights as 5 to 10 per cent.

We have analyzed several preparations of myoglobin, prepared as described above, for iron. These analyses were made by the method described

by Delory (2), with α,α'-dipyridyl as an indicator.   To establish the accuracy of the method, a statistical analysis of its reliability was made. First, twelve standards, to each of which were added 25 γ of iron per 10 ml., were analyzed.   These gave an average iron content of 25.02 γ (σ = 0.80; coefficient of variation = 3.2 per cent).   Secondly, nine samples of 1 ml. each from a single solution of horse heart myoglobin known to be only partially pure were analyzed for iron content and dry weight.   The iron ranged from 6.00 to 6.19 γ, with an average value of 6.10 (σ = 0.2; coefficient of variation = 3.3 per cent).   The dry weights ranged from 1.97 to 2.10 mg. per ml. and averaged 2.03 mg. (σ = 0.14; coefficient of variation = 6 per cent).

Finally eight preparations of myoglobin of different concentrations were analyzed by this method.   The determinations of iron in them were 0.313, 0.317, 0.305, 0.322, 0.352, 0.325, 0.340, and 0.306 per cent, average 0.323 (σ = 0.043; coefficient of variation = 13.3 per cent).

The possibility exists that this value for the iron content of myoglobin is affected by loss of either the heme or globin of denatured molecules.   The solubility and stability of globin from myoglobin are unknown for the conditions of the preparation; however, from knowledge of the relative stabilities of globin and heme of hemoglobin, one would expect the heme to be more stable than the globin.   If this obtains for myoglobin, denaturation would increase the iron content rather than decrease it, and would give values equal to or greater than 0.345 per cent.

The average value for iron content of myoglobin, 0.323 per cent, is in good agreement with the composition calculated from the molecular weights obtained by the sedimentation-diffusion studies (see Table I) discussed above.   It agrees substantially with values for iron in horse hemoglobin found by Zinoffsky (14) and Valer (13).   When used to calculate the molecular weight of myoglobin, 0.323 per cent gives 17,288.   This result agrees well with Polson's (6, 10) determination of the molecular weights of myoglobin, 17,200 and 17,500.

SUMMARY

A method to isolate and purify myoglobin of horse heart is described. The theoretical percentage composition with respect to iron in myoglobin is believed to be nearer 0.323 than the commonly accepted value 0.345. Eight preparations of myoglobin were analyzed.   They were found to have values for iron ranging from 0.305 to 0.352 per cent, with an average of 0.323 per cent.   When used to calculate the molecular weight of myoglobin, 0.323 per cent gives 17,300, which is in good agreement with the results of sedimentation and diffusion studies of myoglobin.

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COMPARATIVE GROWTH ON DIETS CONTAINING TEN AND NINETEEN AMINO ACIDS, WITH FURTHER OBSERVATIONS UPON THE RÔLE OF GLUTAMIC AND ASPARTIC ACIDS*

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In a series of papers extending over many years evidence has been presented regarding the nutritive rôle of each of the recognized components of proteins. The results have demonstrated that ten amino acids are essential dietary components for the rat. The exclusion from the food of any one of these, other than arginine, leads to a profound nutritive failure, loss in weight, and eventual death. Arginine differs in several respects from other constituents of proteins. First, it can be synthesized by the rat (1), but not at a rate commensurate with the needs of the organism for *maximum* growth. Thus, young animals which are deprived of arginine gain much less rapidly than do their litter mates which receive this amino acid (2). Because of this fact, arginine is classified in this laboratory as an essential component of the food. On the other hand, arginine is unique in that it can be replaced in part for growth purposes by proline or glutamic acid, though not by hydroxyproline (3). These relationships are believed to be associated with the ability or inability of the organism to effect the interconversion of the amino acids in question (*cf.* (3–6)).

In connection with the above studies two points require further consideration. The first has to do with the nutritive efficiency of diets in which the ten essential amino acids are virtually the sole sources of nitrogen. The second relates to the precise status of glutamic acid as a growth stimulant *in the presence of arginine*. No data have been presented from this laboratory regarding the comparative growth behavior of animals receiving mixtures of ten and nineteen amino acids. Several years ago, experiments of this nature were conducted under the most favorable conditions then possible. At that time, the conclusion was reached, and stated on several occasions without the submission of evidence (7, 8), that animals upon such simplified diets gain in weight as rapidly as they do when all of the com-

ponents of proteins are supplied preformed. We still believe that this conclusion was correct for the experimental conditions then employed. However, it is not true for the improved dietary régime now used in such studies. As pointed out previously (3), certain moderate deficiencies could not be detected by the growth method until slowly acquired information concerning the nutritive requisites of the rat, and the advent of adequate supplies of crystalline vitamins, enabled us to overhaul thoroughly our basal ration (2).

The primary objective of the present paper was to establish the efficacy, for growth purposes, of mixtures of the essential amino acids. Incidental to these studies, additional information has been obtained concerning the second question mentioned above; namely, the dietary rôle of glutamic acid. Elsewhere (3), attention has been directed to the fact that the growth status of this amino acid is somewhat uncertain. It is less than one-third as effective as arginine when added to a basal ration which is devoid of both of these compounds and the prolines. On the other hand, its addition to a diet carrying sixteen amino acids *including* arginine increases significantly the rate of gain of the experimental subjects. These rather anomalous observations appeared to necessitate additional experiments before one could classify glutamic acid with respect to its function in growth. Accordingly, a considerable number of tests have been conducted for the purpose of determining the *magnitude* of the stimulation exerted by this amino acid when incorporated in diets containing arginine. The results are summarized below.

EXPERIMENTAL

Throughout the investigation, male weanling rats served as the subjects. Each animal was housed in a separate cage, and was permitted to consume food and water *ad libitum*. All tests were continued for 28 days. The amino acids which furnished the nitrogen of the rations were purified invariably until they yielded correct analytical values.

In Table I is presented the composition of three amino acid mixtures which served as the basis of the comparative tests. Mixture XXIII contained all of the amino acids known to be present in proteins except citrulline. The absence of the latter is without effect upon the rate of gain of the rat, as has been shown by numerous experiments in this laboratory. The mixture furnishes each essential in excess quantity, without unnecessary waste, and supports as rapid growth as any preparation we have succeeded in devising. It has been used in hundreds of feeding trials in this laboratory, and is now regarded as our standard. Mixture XXIV was composed of the ten essential amino acids only. Its effective nitrogen, *i.e.* the nitrogen furnished by the *physiologically active* amino acids, was identical with

that of Mixture XXIII. Mixture XXV was analogous in composition to Mixture XXIV except that it contained glutamic acid. By a proportionate adjustment downward in the amounts of the other components, its effective nitrogen was maintained at the same level as that present in Mixtures XXIII and XXIV.

TABLE I

Composition of Amino Acid Mixtures

	Mixture XXIII		Mixture XXIV		Mixture XXV	
	Physio-logically active	As used	Physio-logically active	As used	Physio-logically active	As used
	gm	gm	gm	gm	gm	gm
Glycine..	0 1	0 10				
Alanine.......	0 2	0 40*				
Serine....	0.1	0 20*				
Valine.....	1 0	2 00*	1 32	2 64*	1 14	2 28*
Leucine	1 2	1 20	1 58	1 58	1 37	1.37
Isoleucine...	0 8	1 60*	1 06	2 12*	0 91	1 82*
Cystine..	0 2	0.20				
Methionine.	0 8	0 80*	1 06	1 06*	0.91	0.91*
Threonine....	0.7	1.40*	0 92	1 84*	0 80	1.60*
Phenylalanine..............	1 2	1 20*	1.58	1.58*	1.37	1.37*
Tyrosine..................	0 6	0 60				
Proline..................	0 2	0 20				
Hydroxyproline .	0 1	0 10				
Tryptophan..	0.4	0.40	0.53	0.53	0 46	0.46
Aspartic acid.	0 2	0 40*				
Glutamic "	2.0	2 00			2 00	2.00
Lysine.....	1 2		1.58		1.37	
" monohydrochloride . .		1.50		1 98		1.71
Histidine...	0 7		0 92		0.80	
" monohydrochloride monohydrate		0.95		1.24		1.08
Arginine	0 4		0 53		0 46	
" monohydrochloride .. .		0 50		0.64		0.56
Sodium bicarbonate.........		1.27		1.66		1.44
	12 1	17 02	11.08	16 87	11.59	16.60

* Racemic acids.

In Table II is shown the make-up of Diets 1 to 3 inclusive, containing respectively nineteen, ten, and eleven amino acids. The unknown nitrogen present in the liver extract did not exceed 32 mg. per 100 gm. of food, and consequently could not have contributed significant amounts of glutamic acid or other amino acids. The diets were appropriately supplemented with vitamins. For this purpose, the quantities listed in Table III were thoroughly admixed with each kilo of ration.

The results of the experiments are presented in Table IV. In Series I a comparison was made of the growth of animals upon diets containing ten and nineteen amino acids. For this purpose, thirty-five male rats from five litters were divided as equitably as possible between the two diets. As will be observed, the animals which received the ten essential amino acids showed a mean gain in 28 days of 77.4 ± 1.38 gm., while the positive controls which consumed nineteen amino acids manifested a mean gain of 93.1 ± 1.95 gm. The mean difference (15.7 gm.) is highly significant,

TABLE II

Composition of Diets

	Diet 1	Diet 2	Diet 3
	gm.	*gm.*	*gm.*
Amino acid Mixture XXIII..........................	17.02		
" " " XXIV.........................		16.87	
" " " XXV..........................			16.60
Sucrose..	15.00	15.00	15.00
Dextrin..	59.23	59.38	59.65
Cellu flour......................................	2.00	2.00	2.00
Salt mixture*....................................	4.00	4.00	4.00
Corn oil...	2.00	2.00	2.00
Vitamin A and D concentrate†.....................	0.05	0.05	0.05
Inositol...	0.10	0.10	0.10
Choline chloride.................................	0.20	0.20	0.20
Liver extract‡...................................	0.40	0.40	0.40
	100.00	100.00	100.00

* Jones and Foster (9).
† This contained 65,000 U. S. P. units of vitamin A and 13,000 U. S. P. units of vitamin D per gm.
‡ Wilson's liver powder, 1:20.

and indicates that the simpler amino acid mixture is considerably less effective than the more complex one as a source of nitrogen for growth.

Recently, Womack, and Rose ((3) p. 46) observed that animals upon rations containing L-tryptophan made somewhat better gains than did controls upon like diets carrying the same weight of DL-tryptophan. This finding was unexpected inasmuch as the optical isomers of this amino acid are generally regarded as equally effective in the rat for growth purposes (10, 11). The experiment summarized as Series I in the present paper antedated the observation of Womack and Rose, and involved the use of DL-tryptophan. In view of this fact, it became necessary to repeat the experiment under identical conditions except for the use of L-tryptophan. The results are summarized as Series II in Table IV. In this series three

groups, totaling 101 animals from twenty-seven litters, received diets containing respectively the ten essentials, the ten essentials plus glutamic acid, and nineteen amino acids. Disregarding for the moment the second group

TABLE III

Vitamin Supplements

	Added to each kilo of diet
	mg.
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine hydrochloride	5
Nicotinic acid	5
Calcium *d*-pantothenate	25
p-Aminobenzoic acid	300
α-Tocopherol	25
2-Methyl-1,4-naphthoquinone	2
	γ
Biotin	100

TABLE IV

Growth Effects of Glutamic and Aspartic Acids

The experiments covered 28 days each.

Series No.	No. of animals (males throughout)	Mean gain in weight and probable error of mean	Nature of amino acid mixture
		gm.	
I*	18	77.4 ± 1.38	10 amino acids (Mixture XXIV)
	17	93.1 ± 1.95	19 " " (" XXIII)
II	32	79.1 ± 0.82	10 " " (" XXIV)
	38	91.6 ± 1.07	11 " " (" XXV)
	31	108.4 ± 1.28	19 " " (" XXIII)
III	34	97.2 ± 0.94	18 " " (no glutamic acid)
	33	103.2 ± 1.37	19 " " (Mixture XXIII)
IV	19	109.9 ± 1.28	18 " " (no aspartic acid)
	19	111.6 ± 2.17	19 " " †

* In Series I the amino acid mixtures contained DL-tryptophan; in all other series L-tryptophan was employed.

† This mixture was identical with Mixture XXIII except that it contained 2.0 per cent of L-aspartic acid.

which received eleven amino acids, one will observe that the animals upon the ten essentials showed a mean gain of 79.1 ± 0.82 gm., while the controls upon nineteen amino acids manifested a mean gain of 108.4 ± 1.28 gm. Thus, the mean difference was 29.3 gm., or almost twice as great as that in

Series I. Evidently, a mixture of all of the components of proteins is distinctly superior in nutritive quality to one carrying the ten essentials only. The task of synthesizing ten amino acids *simultaneously* appears to present too great a burden upon the chemical resources of the cells to permit the latter to keep pace with the needs of the organism for *optimum* growth. The astounding fact to be noted is that, in the young rat, the tissues succeed in manufacturing ten of their own constituents at rates which allow bodily gains of 2.8 gm. per day.

The finding that a mixture of ten amino acids is inferior to one containing nineteen is contrary to our observation of 10 years ago with the use of a less satisfactory basal ration. Probably the divergence is attributable to defects in our earlier diets involving constituents other than the amino acids. Thus, inadequate quantities of vitamins, and perhaps of certain non-nitrogenous components of the food, may have limited growth even when all amino acids were present. This possibility is supported by the fact that the best growth then observed was much less than is now obtained regularly.

In this connection, reference must be made briefly to a paper of Albanese and Irby (12) in which the authors report that young rats lose weight upon a diet containing the essential amino acids in approximately the proportion found in casein. The paper is mentioned with reluctance inasmuch as its conclusions have already been refuted by Martin (13) and Kinsey and Grant (14). Albanese and Irby suggest that the inadequacy of their diet may have been due in part to toxic effects of the "unnatural forms of certain amino acids" used in the ration. This explanation is extremely improbable.[1] A more reasonable one is that the diet was deficient in isoleucine. The latter is said to have been supplied as a "*l*-leucine-isoleucine mixture," but no information is presented as to the amount of isoleucine which it contained. In the experiments of Martin, and of Kinsey and Grant, growth occurred invariably upon the diets containing only ten amino acids, but the mean daily gains of the rats were considerably less than those recorded in the present paper. Somewhat earlier, Bauer and Berg (15) had shown that the ten essentials suffice for the slow growth of mice.

The data summarized as Series I and II (Table IV) lend support to the idea expressed elsewhere (3) that L-tryptophan, under certain conditions, permits better growth than the same amount of DL-tryptophan. As will be observed, the two groups of animals which ingested ten amino acids made almost identical mean gains. On the other hand, with the diet containing

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[1] A personal communication from Dr. K. A. J. Wretlind of the Karolinska Institutet, Stockholm, announces that he has obtained weight gains in rats of 0.3 to 0.9 gm. daily upon diets containing 10 to 20 per cent of a mixture of *only* the racemic forms of the ten essential amino acids. His paper is now in press.

nineteen amino acids, the animals which received L-tryptophan showed a mean gain which was 15.3 gm. in excess of that achieved by the subjects which consumed the racemic compound. Thus, the superiority of L-tryptophan is evident only with the ration which otherwise possessed the greater nutritive quality. Statistically the difference is highly significant. Perhaps one may assume from the findings that the inversion of D-tryptophan keeps pace with the needs of the organism for *limited* growth, such as that which occurs when all of the non-essential amino acids must be synthesized concurrently, but is not sufficiently rapid to meet the requirements for *maximum* growth. Possible objections may be raised to this interpretation; but if it should prove to be correct, the behavior of DL-tryptophan will provide another illustration of how the *rate* of a reaction may affect the magnitude of the gain. The problem is being investigated further in this laboratory at the present time.

The data in Table IV are illuminating also with respect to the growth effects of glutamic acid. In Series II, the inclusion of this amino acid in the food increased the mean gain of the subjects from 79.1 ± 0.82 to 91.6 ± 1.07 gm. Thus, the mean difference was 12.5 gm. However, despite the stimulatory action of glutamic acid, the accomplishment of the animals upon eleven amino acids was quite inferior to that of the controls which received nineteen amino acids. The respective gains were 91.6 ± 1.07 and 108.4 ± 1.28 gm., with a mean difference of 16.8 gm. Both increments are highly significant statistically.

Taken by themselves, the findings in Series II are difficult to interpret. Do they imply that glutamic acid has a specific rôle in growth, or do they merely reflect the decreasing synthetic burden upon the animals as the number of preformed amino acids in the food is increased? In order to throw further light upon this question, a comparison was made of the growth of animals upon two diets which were more complete with respect to their amino acid content, and were comparable in composition except that one contained 2 per cent of L-glutamic acid and the other was devoid of this substance. Thus, the general procedure in this experiment was analogous to that followed in most of our previous investigations involving the nutritive rôle of the amino acids. For the purpose in hand, 67 male rats from nineteen litters were divided as equitably as possible into two groups. The positive controls received a ration containing Mixture XXIII. The diet of their litter mates carried a similar mixture from which glutamic acid alone had been excluded. The effective nitrogen content of the two diets was equalized by an appropriate and proportional increase in each of the eighteen amino acids in the second mixture. Thus, the only variable was the glutamic acid content of the food.

The results of the tests are summarized as Series III in Table IV. The

data show that the mean gains of the rats which respectively were deprived of and received glutamic acid were 97.2 ± 0.94 and 103.2 ± 1.37 gm.; hence, the mean difference amounted to 6.0 gm. in favor of the animals which consumed the more complete ration. A difference of this magnitude is of doubtful significance. The ratio of the mean difference to the probable error of the difference is 3.6. This implies that the odds against the difference being due to chance alone are approximately 65 to 1. Most investigators would not be inclined to regard this as convincing. In any event, the exclusion of glutamic acid from a ration containing all of the amino

TABLE V

Classification of Amino Acids with Respect to Their Growth Effects in the Rat

Essential	Non-essential
Lysine	Glycine
Tryptophan	Alanine
Histidine	Serine
Phenylalanine	Cystine*
Leucine	Tyrosine†
Isoleucine	Aspartic acid
Threonine	Glutamic " ‡
Methionine	Proline‡
Valine	Hydroxyproline
Arginine§	Citrulline

* Cystine can replace about one-sixth of the methionine requirement, but has no growth effect in the *absence* of methionine.

† Tyrosine can replace about one-half of the phenylalanine requirement, but has no growth effect in the *absence* of phenylalanine.

‡ Glutamic acid and proline can serve individually as rather ineffective substitutes for arginine in the diet. This property is not shared by hydroxyproline.

§ Arginine can be synthesized by the rat, but not at a sufficiently rapid rate to meet the demands of *maximum* growth. Its classification, therefore, as essential or non-essential is purely a matter of definition.

acids except citrulline does not exert the inhibition in growth one would expect of an indispensable dietary component. Under like conditions, the absence of arginine, the least effective of the essentials, induces a much more profound influence upon the rate of gain in body weight. It seems necessary, therefore, in accordance with our recent tentative suggestion (3), to classify glutamic acid as a dispensable amino acid for the rat despite the fact that it, like proline, can replace in part the arginine requirement of this species.

Series IV of Table IV summarizes, for comparative purposes, the results of similar tests upon the growth effects of aspartic acid. Thirty-eight young rats from eleven litters were divided into two groups of nineteen

animals each, and were placed upon comparable diets except that one contained 2 per cent of aspartic acid and the other was devoid of this amino acid. The nitrogen content of the two rations was equalized as described for the tests of Series III. The data demonstrate clearly that the exclusion of aspartic acid is without effect upon the rate of gain. This is in agreement with the conclusion of Rose and Fierke (16) in earlier experiments involving the use of a much less satisfactory basal ration.

Inasmuch as the present paper is the last from this laboratory dealing specifically with the growth effects of the amino acids in the rat, their final classification into essential and non-essential components of the food is summarized in Table V. This table is a modification of one published elsewhere (17) more than 10 years ago. Since that time, certain interrelationships have been discovered which were not then appreciated. Furthermore, during the same interval marked species differences have come to light. No longer is one warranted in referring to amino acids as dispensable or indispensable without designating the species in which the tests were made, and indicating the criterion used as the basis of the classification. The conclusions presented in Table V apply to the growth of the rat only.

SUMMARY

Experiments have been conducted upon a relatively large number of young rats to determine the comparative efficiency for growth purposes of mixtures of ten and nineteen amino acids. Contrary to our observations of more than a decade ago, involving the use of a less satisfactory basal ration, the results demonstrate that the simpler mixture possesses a lower nutritive value as measured by the relative gains in weight of the subjects. During periods of 28 days, animals which receive the ten essentials only, in an otherwise adequate diet, gain approximately 70 to 75 per cent as much as their litter mates which consume nineteen amino acids.

Further experiments upon the rôle of glutamic acid in growth indicate that the addition of this amino acid to a ration containing the ten essentials exerts a significant stimulatory effect upon the rate of gain, but that its removal from a diet containing nineteen amino acids is followed by a very slight inhibition which statistically is of doubtful significance. Under the latter conditions, its influence, if any, upon growth is certainly much less than that manifested by arginine. In view of these facts, glutamic acid is classified as a dispensable dietary component for the rat.

In confirmation of an earlier investigation from this laboratory, the exclusion of aspartic acid from the food does not affect the growth of the rat.

A final classification of the amino acids with respect to their rôle in the growth of the rat is presented.

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ENZYMATIC DEHALOGENATION OF CERTAIN BROMINATED AND CHLORINATED COMPOUNDS

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The dehalogenation *in vivo* of brominated aliphatic hydrocarbons such as methyl bromide (1) and bromochloromethane (2) has been demonstrated by recovery of inorganic bromide from blood and urine. The intracellular formation of bromide and methyl alcohol from methyl bromide has been advanced as an explanation for its prolonged toxic effect (3). Evidence for the splitting of chlorinated aliphatic hydrocarbons has not been obtained. Thus, methyl alcohol could not be detected in the blood of animals exposed to methyl chloride (4). In the present investigation it was found that protein fractions from liver catalyzed the hydrolysis of bromochloromethane (CH_2BrCl), dibromomethane, and dichloromethane to hydrogen ion, halide ion, and formaldehyde. The enzyme system was activated by cyanide and sulfhydryl compounds.

Methods

Preparation of Tissues—Slices of tissue approximately 0.3 mm. thick were cut free-hand and immersed in Krebs-Ringer-phosphate solution (5). Homogenates were prepared according to Potter and Elvehjem (6). Extracts were made by centrifuging homogenates for 10 minutes at 11,000 R.P.M. in an angle centrifuge at 2°. The solution used for preparing homogenates and extracts was similar to that of Buchanan, Hastings, and Nesbett (7).

Measurement of Enzymatic Dehalogenation—Inorganic bromide formed in an hour from brominated hydrocarbons was proportional to the concentration of tissue. The reaction was stopped by addition of trichloroacetic acid (final concentration 5 per cent). Bromide was determined by Friedman's procedure (8) after removal of organic brominated compounds by aeration for 15 minutes at 40°.

Assay for enzymatic activity was also carried out by measuring the rate of CO_2 evolution from a bicarbonate buffer due to the liberation of hydrogen

ion from halogenated compounds. Warburg flasks were gassed in pairs for 8 minutes, at a rate of 100 ml. per minute, with 95 per cent N_2-5 per cent CO_2. The gas stream was first bubbled through 500 ml. of fluid containing the desired concentration of halogenated compound. By this procedure the concentration of volatile substrate in the reaction vessels was 80 to 95 per cent of that in the reservoir wash bottle (9). After a 5 minute equilibration period CO_2 evolution was measured for 20 minutes, during which time the rate was linear.

Determination of Formaldehyde—The incubation mixture was distilled with acid and the chromotropic acid procedure for formaldehyde was applied to the distillate (10). Fraenkel-Conrat *et al.* (11) have pointed out that proteins rich in indole groups will bind formaldehyde irreversibly even

TABLE I

Per Cent Recovery of Formaldehyde Added to Rat Liver Extracts

Formaldehyde, usually 120 γ, was added to 2.5 ml. of liver extract, equivalent to 375 mg. of liver. The volume was made to 10 ml., after which the containers were stoppered and kept at 25° for 15 minutes. Then acid was added and the volume was made up to 20 ml. Distillation was carried nearly to dryness. With steam distillation 25 ml. of distillate were collected.

	H_2SO_4		HCl		CCl_3COOH	
	20 per cent (volume)	10 per cent (volume)	20 per cent (volume)	10 per cent (volume)	20 per cent (weight)	10 per cent (weight)
Distillation.............	51.2 (5)*	87.1 (2)	82.7 (3)	85.3 (3)	96.0 (7)	92.1 (4)
Steam distillation......	82.3 (5)	97.4 (3)	92.9 (3)	90.3 (3)	93.4 (3)	90.1 (4)

* The figures in parentheses indicate the number of determinations whose result are averaged.

under conditions of combined acid hydrolysis and distillation. Table I indicates that formaldehyde could be recovered in satisfactory amounts when added to liver extracts, provided that a suitable choice of acid and method of distillation was made. It should be noted that the commonly used procedure of distillation with sulfuric acid gave low recoveries.

Preparations Used—The CH_2BrCl[1] was a fraction distilling in a 4 foot column at 67–68°, with an index of refraction n_D^{26} of 1.4796 and a density at 25° of 1.930. The compounds CH_2Br_2, CH_2Cl_2, $CHBr_3$, $CH_2Cl \cdot CH_2Cl$, and $CHCl_3$ were also redistilled. The other compounds were Eastman Kodak products used without purification.

[1] This compound was obtained from the Michigan Chemical Corporation, St. Louis, Michigan. The commercial sample had a distillation range of 64.8–67.5°.

Results

Dehalogenation of Aliphatic Halogenated Hydrocarbons by Tissue Preparations—Table II shows that slices of rat liver, kidney, and spleen catalyzed the formation of bromide from CH_2BrCl. Certain other tissues were relatively ineffective under these conditions. Homogenates of liver were active but those of kidney showed greatly reduced or no activity. The cleavage of CH_2BrCl by rat liver homogenate was 3 times as rapid in nitrogen as in air. It was completely inhibited by 10^{-3} M $HgCl_2$ and by 5 minutes incubation at 60°. 50 per cent inhibition was caused by 10^{-2} M NaF and 30 per cent inhibition by 10^{-4} M $HgCl_2$.

TABLE II

Formation of Inorganic Bromide from CH_2BrCl by Rat Slices from Various Organs

Slices weighing 500 mg. and about 0.3 mm. in thickness were incubated with shaking at 37°. Incubation time, 1 hour; gas phase, air; immersion fluid, 10 ml. of Krebs-Ringer-phosphate, pH 7.4; concentration of CH_2BrCl in liquid phase, 0.011 M. The results are averages of at least two separate assays.

Organ	Inorganic bromide per gm. wet tissue per hr.
	mg.
Kidney	1.09
Liver	0.71
Spleen	0.26
Brain	0.08
Heart	0.06
Diaphragm	0.06

Table III indicates the relative amounts of bromide and formaldehyde resulting from incubation of CH_2BrCl and CH_2Br_2 with rat liver extract. The results are consistent with the following equations:

$$CH_2BrCl + H_2O = 2H^+ + Br^- + Cl^- + HCHO$$
$$CH_2Br_2 + H_2O = 2H^+ + 2Br^- + HCHO$$
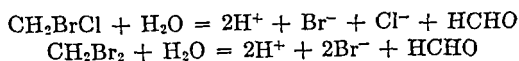
In the case of CH_2Cl_2 no exact comparisons were made but the accumulation of formaldehyde was of the same order of magnitude as hydrogen ion production measured manometrically. In several experiments with CH_2BrCl formaldehyde was determined colorimetrically and also gravimetrically as the dimedon derivative (12). Satisfactory agreement was obtained and the crystalline dimedon compound had the same melting point and mixed melting point as an authentic sample.

The rate of CO_2 evolution from a bicarbonate buffer due to fixed acid production from CH_2BrCl was proportional to the concentration of tissue. This is shown in Fig. 1. The ordinates of Fig. 1 represent uncorrected

values for CO_2 because volatile substrate was disappearing at a rate proportional to CO_2 formation. The true values are somewhat larger than those indicated.

Fig. 2 shows that with optimal substrate concentrations CH_2BrCl was dehalogenated about 4 times as rapidly as CH_2Br_2 or CH_2Cl_2. Chloroform was acted upon slowly and bromoform was not attacked. Several ethane derivatives were tested either manometrically or by measuring inorganic bromide. With C_2H_5Br, $CH_2Br \cdot CH_2Br$, $CH_2Cl \cdot CH_2Br$, and $CH_2Cl \cdot CH_2Cl$ the rate was somewhat slower than that observed with CH_2Br_2.

TABLE III

Comparison of Amounts of Bromide and Formaldehyde Formed from
CH_2BrCl and CH_2Br_2 during Incubation with Rat Liver Extract

Anaerobic incubation was carried out at 37°, for 60 minutes with CH_2BrCl and 90 minutes with CH_2Br_2. Each flask contained 10 ml. of fluid, including liver extract equivalent to 375 mg. of fresh liver. The medium contained K^+, 0.13 M; Mg^{++}, 0.01 M; Cl^-, 0.15 M; Na^+, 0.03 M; phosphate, pH 7.4, 0.02 M. The concentration of substrate was 0.022 M.

	Experiment No.	Bromide production	Formaldehyde production	$\frac{Br^-}{HCHO}$
		micromoles	*micromoles*	
CH_2BrCl	1	5.4	5.2	1.0
	2	6.5	6.4	1.0
	3	9.3	8.6	1.1
Average......				1.05
CH_2Br_2	1	7.1	2.8	2.5
	2	6.5	5.3	1.2
	3	4.0	1.9	2.1
	4	3.7	1.7	2.2
Average......				2.0

Activation by Cyanide and Sulfhydryl Compounds—Crude liver extracts aged at 0°, dialyzed extracts, and ammonium sulfate fractions required activation by cyanide and either glutathione or cysteine. Table IV indicates that with the concentrations tested both glutathione and cyanide were needed for the greatest effect. Irving, Fruton, and Bergmann (13) have recorded some interesting experiments with papain and cathepsin, whose requirements for activation are somewhat similar to those recorded here.

Purification of Enzyme Activity—A 5-fold purification of the crude rat liver extracts by ammonium sulfate fractionation could be demonstrated. A fraction was precipitated between 0.45 and 0.55 saturation. This frac-

tion had a pH optimum in the range 7.1 to 7.4. At pH 5.1 and 8.5 enzymatic activity was lost after several hours at 2°. At pH 7.4 and 2° the activity decreased by one-third over a period of several weeks.
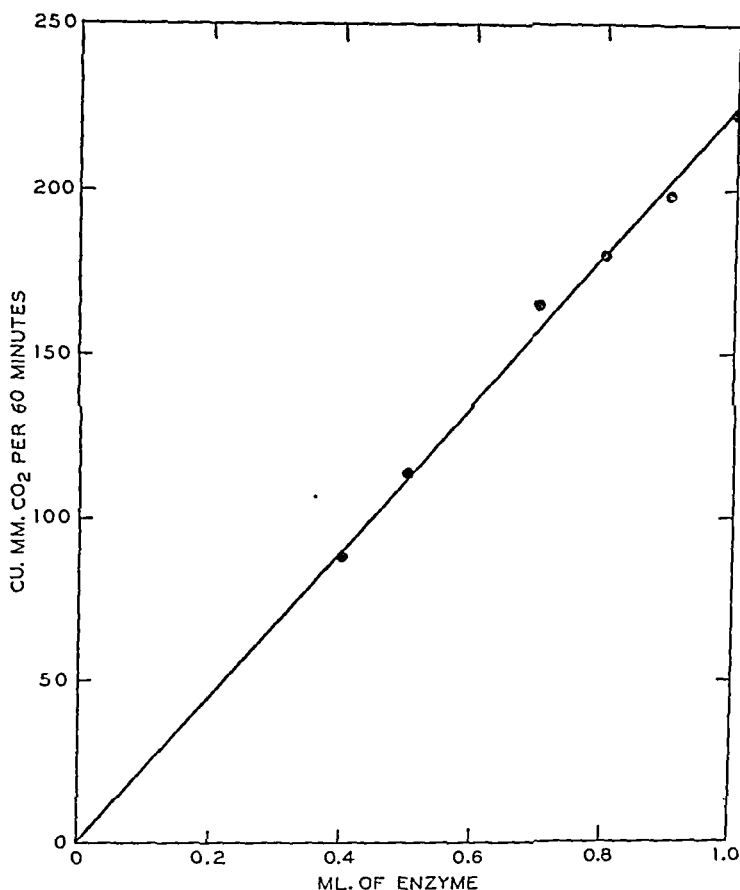
FIG. 1. Relationship between reaction velocity and concentration of enzyme. An ammonium sulfate fraction of rat liver with 10 mg. of protein per ml. was used. The Warburg flasks also contained K^+, 0.05 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; CN^-, 0.01 M; HCO_3^-, 0.017 M; glutathione, 1.7×10^{-3} M; CH_2BrCl, 0.022 M; total volume, 3 ml.

Effect of Exposure of Rats to CH_2BrCl on Enzymatic Activity of Liver—
Adult male rats were exposed 6 to 7 hours daily, 5 days a week, to an atmosphere containing 1,000 parts per million of CH_2BrCl. After twenty to thirty exposures the rats were killed and homogenates of their livers were incubated in air with CH_2BrCl. Seventeen of these preparations formed an
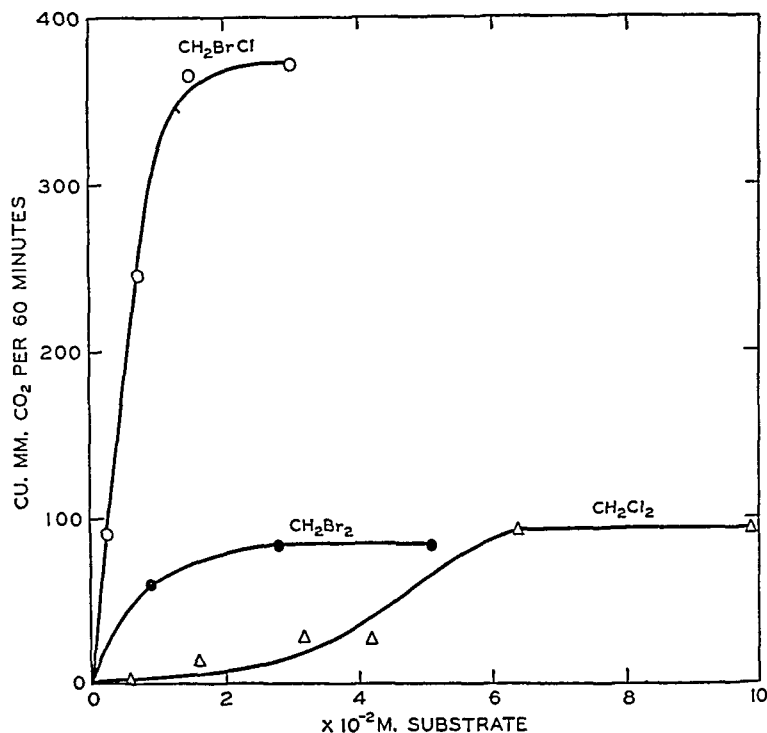
FIG. 2. Comparison of rates of enzymatic hydrolysis of different substrates. Each flask contained 1 ml. of crude rat liver extract with a protein content of 60 mg. The flasks also contained K^+, 0 05 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; CN^-, 0.01 M; HCO_3^-, 0.017 M; glutathione, 1.7×10^{-3} M; CH_2BrCl, 0.022 M; total fluid volume, 3 ml.

TABLE IV

Requirements of Ammonium Sulfate Fraction for Glutathione and Cyanide in Order to Catalyze Hydrolysis of CH_2BrCl

The Warburg flasks contained a fluid volume of 3 ml.; K^+, 0.04 M; Cl^-, 0.048 M; Na^+, 0.017 M; Mg^{++}, 0.004 M; HCO_3^-, 0.017 M; CH_2BrCl, 0.022 M; enzyme solution, 1 ml., with 9 mg. of protein.

Concentration of GSH	Concentration of CN^-	Enzyme activity
M	*M*	*c.mm. CO_2 per hr.*
0	0.01	0
2.1×10^{-4}	0.01	74
2.1×10^{-3}	0.01	195
2.1×10^{-4}	0	0
2.1×10^{-3}	0	46
1.8×10^{-2}	0	26

average of 1.32 mg. of bromide per gm. of wet tissue per hour compared with a figure of 0.81 for homogenates from fourteen unexposed control rats. The difference was statistically highly significant ($P < 0.001$ with Fisher's t method (14)).

Formaldehyde was demonstrated in the livers of rats killed after an hour of deep narcosis with CH_2BrCl. The concentration was only 1.1 mg. per 100 gm. of liver. This may be due to oxidation *in vivo* of most of the formaldehyde resulting from hydrolysis of CH_2BrCl.

SUMMARY

1. The enzymatic dehalogenation of CH_2BrCl, CH_2Br_2, CH_2Cl_2, $CH_2Br \cdot CH_2Br$, $CH_2Cl \cdot CH_2Cl$, $CHCl_3$, C_2H_5Br, and $CH_2Cl \cdot CH_2Br$ has been demonstrated in liver extracts. The products of reaction with CH_2BrCl, CH_2Br_2, and CH_2Cl_2 were formaldehyde, halide ion, and hydrogen ion.

2. The enzyme system required activation by cyanide and either glutathione or cysteine.

3. A 5-fold purification was achieved by fractionation of liver extracts with ammonium sulfate.

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HEPATORENAL FACTORS IN CIRCULATORY HOMEOSTASIS

IX. THE IDENTIFICATION OF THE HEPATIC VASODEPRESSOR SUBSTANCE, VDM, WITH FERRITIN*

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Earlier studies from this laboratory (1) have revealed the regular participation in experimental shock of two hitherto undescribed vasotropic principles, a vasoexcitor material (VEM) of renal and a vasodepressor material (VDM) of hepatic origin. The renal vasoexcitor appeared during the initial compensatory or hyperreactive phase of shock, the hepatic vasodepressor during the subsequent decompensatory or hyporeactive phase. The effect of the renal vasoexcitor on the terminal vascular bed of an animal in shock was to increase spontaneous vasomotion and enhance the reactivity of the terminal arterioles and precapillary sphincters to the topical application of epinephrine. The hepatic vasodepressor, on the other hand, brought about a reduction of vasomotion and a depression of the reactivity of these terminal muscular vessels to epinephrine. These principles were detected in both blood and tissue extracts by the vascular effects which they induced when injected intravenously into anesthetized normal rats whose mesoappendix was exposed for direct visualization. Enhancement or depression of the reactivity of the blood vessels to the topical application of epinephrine permitted the differentiation between the vasoexcitor and vasodepressor principles (2).

The present study is concerned with the purification and chemical characterization of VDM of hepatic origin and with its relationship to the VDM present in the blood during the hyporeactive phase of shock. Although our studies have shown that a vasodepressor material of comparable activity is also formed by skeletal muscle and spleen during the shock syndrome, attention in this study has been largely focused on the VDM of hepatic origin.

With the progressive concentration of VDM activity in extracts of beef,

dog, rat, and human liver, preparations were obtained which were increasingly concentrated with respect to the ratio of iron to nitrogen. The iron component had the characteristics of colloidal ferric hydroxide. The possibility was therefore explored that hepatic VDM might be identical with ferritin, an unusual iron-protein first crystallized by Laufberger (3) and shown to be present in both liver and spleen. This identification was established by a combination of chemical and immunochemical procedures. It was further found that ferritin (or apoferritin) was identical with naturally occurring VDM, present in the liver and blood during hyporeactive shock and in the blood during the chronic stage of experimental renal and human essential hypertension.

<div align="center">EXPERIMENTAL</div>

Our previous studies (1) have established the conditions *in vivo* and *in vitro* for the formation and inactivation of VDM. These have been listed in Table I. This information made it possible to set up appropriate conditions not only for obtaining maximal yields of hepatic VDM for chemical fractionation, but also for clarifying, by immunochemical procedures, the relationship of the purified VDM of hepatic origin to naturally occurring VDM.

The method of preparation of active VDM solutions for purposes of chemical fractionation was based on Reaction 4, Table I. The rat mesoappendix technique was utilized for the determination of the potency of VDM during the process of fractionation (2). The test sample was diluted with 0.9 per cent saline until the intravenous injection into a test rat of 0.5 ml. resulted in an inhibition of the epinephrine response which lasted from 20 to 40 minutes. This depression of epinephrine reactivity is similar to that induced by 0.5 ml. of dog plasma removed during the irreversible phase of hemorrhagic shock. These bioassays were conducted under the supervision of our associate, Dr. B. W. Zweifach.

Concentration of Beef Liver VDM—Large scale preparation of VDM was carried out with beef liver in the laboratories of Eli Lilly and Company, Indianapolis, under the direction of Dr. E. D. Campbell. The liver, collected immediately after slaughter of the animal, was packed in insulated cans and maintained at approximately body temperature for 2 hours. This procedure was effective in maintaining anaerobic conditions for the greatest proportion of the tissue. The liver was then sliced with an electric slicing machine as thin as feasible (about 2 mm.). The slices were washed by gentle mixing with 5 volumes of 0.9 per cent saline and the extract clarified in a Sharples centrifuge. This extract corresponds to Fraction A in Table II. Table II gives the method used for the concentration of VDM. The procedure was carried through Step 3 by Dr. Campbell, and the

dialyzed solution, which usually represented a concentrate from 60 kilos of liver, was lyophilized and sent to our laboratory for further fractionation. Steps 4 and 6 lowered the total recoverable activity but were nevertheless advantageous, since they removed appreciable amounts of inactive material. The degree of concentration of VDM activity, by the method outlined in Table II, varied considerably from one preparation to another (see Table III).

TABLE I

Factors Governing Origin and Inactivation of VDM

For studies *in vitro* liver slices (dog, rabbit, rat) were incubated with 5 volumes of Ringer-phosphate, pH 7.4, at 37.5° for 2 hours. The centrifuged clear solution was injected into the rat to test for VDM activity. A neutral test signified absence of any effect on the reactivity of the blood vessels of the mesoappendix to topical epinephrine. Anaerobic liver slices were prepared by incubation of normal liver slices in Ringer-phosphate in N_2 for 2 hours. The slices were removed from the solution, washed with cold saline to remove any adhering VDM, and then used for further incubation.

	Experimental conditions	Rat assay
In vivo	Reaction 1. Plasma, irreversible shock (hyporeactive stage)	VDM
	" 2. Liver, irreversible shock (hyporeactive stage)	"
In vitro	" 3. Normal liver slices in O_2	Neutral
	" 4. " " " " N_2	VDM
	" 5. Anaerobic liver slices in O_2	"
Inactivation (*in vitro*)	" 6. Normal liver slices + VDM in O_2	Neutral
	" 7. " " " + " " N_2	VDM
	" 8. Anaerobic liver slices + VDM in O_2	"
	" 9. Liver slices, irreversible shock, + VDM in O_2	"

The properties of Fraction B, Table II, were those of proteins. 50 per cent of the total nitrogen of an acid hydrolysis mixture was accounted for by humin, ammonia, glutamic acid, phenylalanine, tyrosine, arginine, and histidine (4). The concentrated beef liver VDM gave negative Molisch and pentose tests and contained 0.1 per cent phosphorus. The straw-yellow VDM solutions contained protein-bound iron in variable concentrations. Addition of $Na_2S_2O_4$ and pyridine yielded no absorption bands of hemochromogens. The iron was therefore not present as a porphyrin complex.

An electrophoretic study[1] of one beef liver VDM preparation (Fraction

B) revealed a mixture of at least two protein components with different mobilities. Another such fraction was run in the ultracentrifuge[1] at 34,000 R.P.M. for 30 minutes. The original solution, before centrifugation, contained 0.01 mg. of iron per mg. of nitrogen. After centrifugation, most of the color had concentrated in the form of a dark brown pellet at the bottom of the tube. It was separated and redissolved in phosphate buffer, pH 7.4. This solution was found to contain 0.17 mg. of iron per mg. of nitrogen and contained the VDM activity. The almost colorless supernatant was very low in iron and was devoid of VDM activity.

Concentration of Dog Liver VDM—Dog liver VDM was prepared in our laboratory from two sources: from slices of liver obtained from dogs in hyporeactive shock and washed with saline, and from normal dog liver

TABLE II

Concentration of Liver VDM Activity

The steps outlined are for the preparation of VDM from beef liver. Steps 4 and 6 were omitted for the preparation of VDM from dog, horse, human, and rat.

Step 1. Extract anaerobic liver slices with 5 volumes saline; centrifuge (Fraction A)
"   2. Adjust filtrate to pH 5.0 with 1 N HCl; heat in water bath to 80–85°; filter; discard ppt.
Step 3. Concentrate filtrate *in vacuo* approximately 20-fold; dialyze against running tap water
Step 4. Saturate with NaCl; centrifuge ppt. and redissolve in water; dialyze
"   5. Make 50% saturated with (NH₄)₂SO₄; centrifuge ppt. and redissolve in water; repeat 4 times; dialyze
Step 6. Saturate with NaCl; centrifuge ppt.; redissolve and repeat; dialyze
"   7. Make 30% saturated with (NH₄)₂SO₄; centrifuge ppt. and repeat; dialyze (Fraction B)

slices incubated in nitrogen for 2 hours at 37.5° (Reactions 2 and 4, Table I). Maximal concentration of VDM activity was obtained by using the method outlined in Table II, except for the omission of Steps 4 and 6. Repeated fractionation with varying concentrations of (NH₄)₂SO₄ yielded a fraction with 1.12 mg. of iron per mg. of nitrogen, which gave a positive VDM test in concentrations of 0.0005 γ of nitrogen per 0.5 ml. of solution. In this fraction as well as in those obtained from beef liver, the iron was firmly bound to the protein, from which it was not removed by dialysis in the presence of 0.1 M phosphate buffer, pH 7.4, 0.05 M NaCN, 1 M acetate buffer, pH 4.6, or 0.01 N HCl. The iron could be removed from the protein by dialysis in acetate buffer, pH 4.6, in the presence of Na₂S₂O₄. Under these conditions the addition of α,α'-dipyridyl gave rise to the pink color of the ferrous-dipyridyl complex.

Table III presents the results of a comparison of the VDM activity with

the iron and nitrogen content of a number of VDM preparations from beef, dog, horse, rat, and human liver. There was no relation between VDM activity and the N content. However, within the experimental error of the rat assay method, a good correlation was found between the iron content and VDM activity.

Relation of VDM to Ferritin—The presence of iron in all fractions with VDM activity as well as the chemical nature of the iron-protein linkage led to a consideration of the possible identity of VDM with ferritin. The

TABLE III

Correlation of VDM Activity and Fe Content

Source of liver	Preparation No.	Fe Content	VDM activity in terms of	
			Nitrogen	Iron
		mg. Fe per mg N	γ	γ
Beef	1072-3	0.004	0.05	0 0002
"	1070	0.009	0.02	0 0002
"	1567	0.011	0.03	0.0003
"	1059	0.016	0.03	0.0005
"	1085	0.016	0.05	0.0008
"	1084	0.068	0.004	0.0003
"	EQ*	0.604	0.001	0.0006
Human	1	0.24	0.001	0.0002
Rat	1	0.38	0.001	0.0004
"	2	0.53	0.001	0.0005
Horse	1	0.78	0.001	0.0008
Dog	2	0.311	0.001	0.0003
"	1	0.354	0.001	0.0004
"	10	0.500	0.0006	0.0003
"	3*	1.120	0.0004	0.0004

* These preparations were obtained by repeated fractionation of Fraction B, Table II, with concentrations of $(NH_4)_2SO_4$ varying from 20 to 30 per cent of saturation.

exploration of this possibility was facilitated by the fundamental studies of Granick (5) on crystalline ferritin.

The addition to preparations of dog liver VDM (such as Preparation 3, Table III) of a 20 per cent solution of $CdSO_4$ to a final concentration of 5 per cent resulted in the deposition of dark brown crystals typical of ferritin (Fig. 1). The presence of ferritin in VDM preparations being established, it was then necessary to determine whether ferritin was the principle responsible for the vasotropic activity of these preparations.

To permit a comparison of the chemical and vasotropic properties of VDM concentrates with ferritin, crystalline ferritin was prepared from

horse spleen and liver, dog liver, and human liver obtained at autopsy. The method was essentially that of Granick (6) with some modifications.[2] Prior to the precipitation of crude ferritin by 50 per cent saturation with $(NH_4)_2SO_4$, as outlined by Granick, the solution was adjusted to pH 4.6 with 50 per cent acetic acid and allowed to stand for several hours at room temperature or overnight in the refrigerator. The precipitate which formed was centrifuged and discarded. The ferritin was then crystallized with $CdSO_4$, redissolved in 2 per cent $(NH_4)_2SO_4$, and recrystallized four
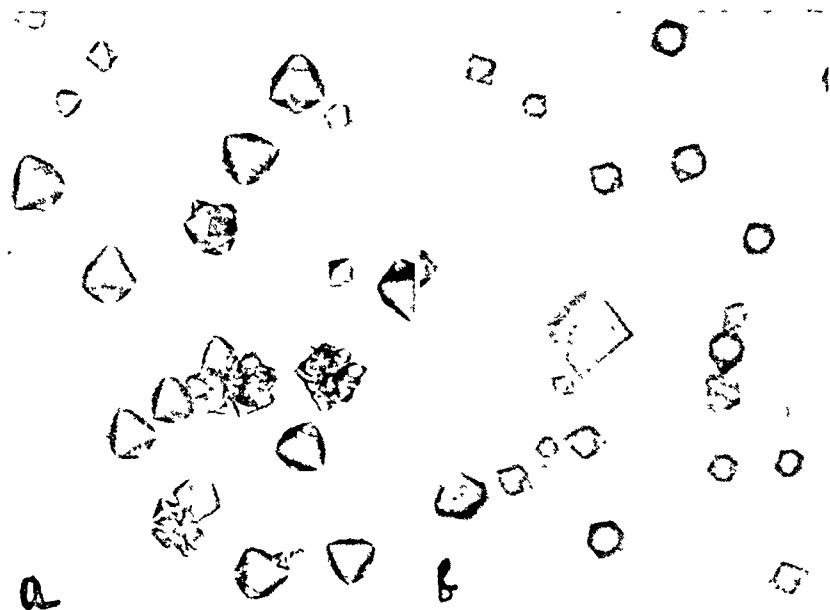
FIG. 1. Crystals (a) of dog liver ferritin prepared according to Granick (6) and (b) from a highly concentrated dog liver VDM solution.

times. It was next dissolved in 2 per cent $(NH_4)_2SO_4$ and dialyzed against running tap water overnight to remove most of the cadmium. The clear solution was then treated with an equal volume of saturated $(NH_4)_2SO_4$ and the precipitate recovered by centrifugation. It was redissolved in water and the reprecipitation with $(NH_4)_2SO_4$ repeated three times. This procedure served to reduce further the Cd content of the final ferritin solution. The contaminating $(NH_4)_2SO_4$ was removed by exhaustive dialysis and the concentrated ferritin solutions stored in the refrigerator with toluene as the preservative. It was found that freezing of such solutions should be avoided, since some insoluble ferritin appeared on thawing, which did not redissolve completely.

[2] Agner, K., personal communication.

A typical ferritin preparation from horse spleen contained 20.7 per cent iron, 11.0 per cent nitrogen, 1.29 per cent phosphorus, and 0.23 per cent cadmium. Thus, it contained 1.88 mg. of iron per mg. of nitrogen.

The visible light absorption spectra of purified crystalline horse spleen ferritin and a dog liver VDM preparation are shown in Fig. 2. Also shown is the absorption spectrum of a colloidal ferric hydroxide solution prepared by heating a dilute solution of ferric chloride to 100°. The three curves
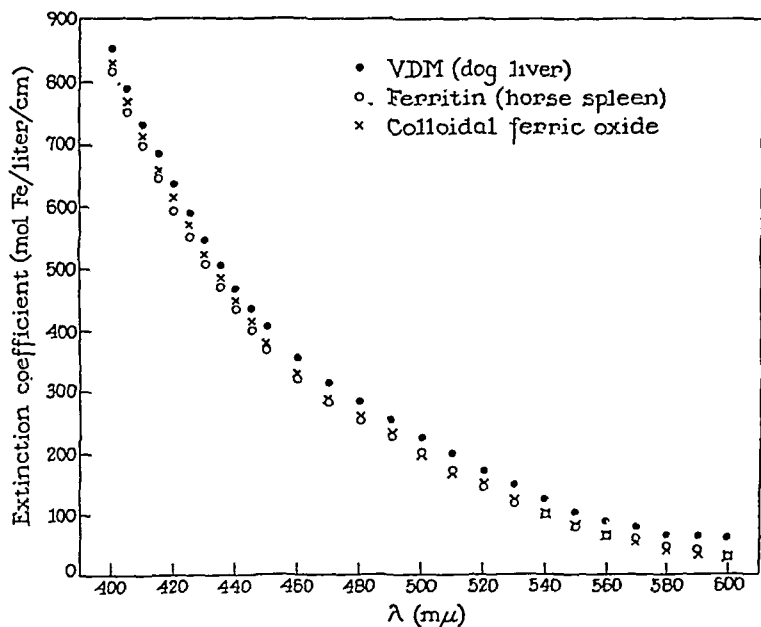
FIG. 2. Visible light absorption spectra of solutions of dog liver VDM, crystalline horse spleen ferritin, and colloidal ferric hydroxide, determined by means of the Beckman spectrophotometer. ·

are almost identical when the wave-length is plotted against the extinction coefficient calculated on the basis of iron content.

Ultracentrifuge determinations[1] were carried out on a preparation of horse spleen ferritin and on a highly concentrated VDM solution prepared from the liver of dogs in the irreversible phase of hemorrhagic shock. The ferritin was purified without the use of CdSO$_4$, by means of alcohol precipitation at low temperatures.[2] The ferritin solution, which contained 2.10 mg. of iron per mg. of nitrogen, was centrifuged at 24,000 R.P.M. The deep brown color of the solution was associated with a rapidly sedimenting component, corresponding to the aggregated apoferritin-iron hydroxide complex as reported by Rothen (7). The VDM solution ˮʰⁱᶜʰ

contained 1.17 mg. of iron per mg. of nitrogen, was centrifuged in a similar manner.   The deep brown material sedimented at a rate similar to that of the ferritin boundary previously obtained.   However, there was present, in addition, an equal amount of a colorless component of a much lower molecular weight, the boundary of which did not quite detach itself from the meniscus after centrifugation for 36 minutes at 24,000 R.P.M.   The VDM solution therefore contained an iron-protein fraction with sedimentation characteristics similar to that of ferritin.   This fraction constituted approximately one-half of the total protein.

The pure ferritin solutions were active at 0.0005 γ of nitrogen per 0.5 ml. in the test rat.   This is equal in physiological activity to the most concentrated of the hepatic VDM preparations (Table III).

In order to determine whether the vasodepressor activity of ferritin was associated with the iron or protein moiety, the activity of iron-free crystalline apoferritin was investigated.   The apoferritin was prepared according to Granick and Michaelis (8) and further purified by four precipitations at 50 per cent saturation with ammonium sulfate.   The purified apoferritin contained 16.2 per cent nitrogen, 0.05 per cent phosphorus, and 0.16 per cent cadmium.   It was free of iron.   Apoferritin gave a positive VDM test with 0.0005 γ of nitrogen, a potency similar to that of crystalline ferritin.   VDM activity was therefore associated with the protein moiety of ferritin.   The VDM activities of a number of ferritin and apoferritin preparations are recorded in Table IV.   Two fractions, Nos. 1162A and 1162F, Table IV, were precipitated from a ferritin preparation, No. 1162, by fractionation with varying concentrations of $(NH_4)_2SO_4$.   Fraction 1162A, precipitated at 23 per cent saturation with $(NH_4)_2SO_4$, contained more iron (2.20 mg. of iron per mg. of nitrogen), whereas Fraction F, precipitated at 34.6 to 40 per cent saturation with $(NH_4)_2SO_4$, contained less iron (0.93 mg. of iron per mg. of nitrogen) than the original ferritin solution, Preparation 1162 (1.88 mg. of iron per mg. of nitrogen).   Also shown are two fractions, Nos. 1162X and 1162Y, prepared by the centrifugation of ferritin Preparation 1162 at 13,000 R.P.M. for 30 minutes at 5° in a Sorvall centrifuge.   Fraction 1162X was pipetted from the top of the resultant solution and contained 1.28 mg. of iron per mg. of nitrogen, whereas Fraction 1162Y was recovered from the bottom of the tube as a dark brown pellet and redissolved in water.   It contained 2.49 mg. of iron per mg. of nitrogen.   All four fractions thus prepared, together with the original ferritin solution from which each had been obtained, had identical VDM activities on the basis of their protein or nitrogen content, although their iron content varied.   All gave typical ferritin crystals with $CdSO_4$.

Inactivation of VDM Activity of Ferritin after Aerobic Incubation with

Liver Slices—In order to ascertain the possible identity between ferritin and naturally occurring VDM, a variety of experiments were carried out by means of which such an identity could be established. A characteristic of naturally occurring VDM is its inactivation by normal liver slices on aerobic incubation *in vitro* at 37.5° (9) (Reaction 6, Table I). This is true for VDM in the blood and in saline washes of the liver of dogs in irreversible hemorrhagic shock, and for VDM resulting from the anaerobic

TABLE IV

VDM Activity of Ferritin and Apoferritin

Source of preparation	Fe content	VDM activity in terms of	
		Nitrogen	Iron
Ferritin			
	mg. Fe per mg. N	$\gamma \times 10^{-4}$	$\gamma \times 10^{-4}$
Horse spleen, Preparation 1166	1.27	5	6
" " " 4	1.31	5	7
" " " 2	1.73	5	9
" " " 1162	1.88	5	9
" " Fraction 1162F	0.93	5	5
" " " 1162A	2.20	5	11
" " " 1162X	1.28	5	6
" " " 1162Y	2.49	5	12
Dog liver, Preparation 2	1.24	5	6
" " " 3	1.30	5	7
" " " 1	1.68	5	8
Human liver, Preparation 1	1.09	5	5
Horse " " 1	1.22	5	6
Apoferritin			
Horse spleen, Preparation 1	0	5	0
Dog liver, Preparation 1	0.06	1	0.06
" " " 2	0.04	5	0.2
Human liver, Preparation 1	0.02	5	0.1

incubation of normal liver slices. However, VDM cannot be inactivated by normal liver slices which have been exposed to a previous anaerobic incubation for 2 hours (Reaction 8, Table I).

A series of experiments was performed, in collaboration with Dr. Zweifach, to determine whether the VDM activity of ferritin could be inactivated under these conditions. The results are presented in Table V. The VDM activity of 0.0005 γ of ferritin nitrogen, which is equivalent to the VDM activity of 0.5 ml. of plasma from dogs in irreversible shock, was completely inactivated by incubation with normal dog liver slices in oxygen.

Inactivation also occurred with twice this concentration, or 0.001 γ of ferritin nitrogen. When the liver slices were kept anaerobic for 2 hours prior to aerobic incubation with ferritin, no inactivation of the VDM activity due to ferritin occurred. In these respects the behavior of ferritin and that of naturally occurring VDM were identical.

Identification of Hepatic VDM with Ferritin by Immunochemical Procedures—In order to provide more specific evidence of the identity of the VDM in concentrated beef and dog liver fractions with ferritin, quantitative immunochemical techniques were employed.[3] Horse spleen ferritin, recrystallized four times and further purified as described previously, was

TABLE V

Inactivation of VDM Activity of Ferritin by Liver Slices

Liver slices were incubated for 2 hours with 5 volumes of Ringer-phosphate at pH 7.4, or with ferritin dissolved in Ringer-phosphate, pH 7.4. Anaerobic liver slices were prepared by incubating for 2 hours with Ringer-phosphate in N_2, washed with cold saline, and then treated with the appropriate solution. The ferritin solution used for incubation contained 0.005 γ of ferritin nitrogen per 0.5 ml. It was tested as such, diluted 1:5 (0.001 γ of ferritin nitrogen per 0.5 ml.) and diluted 1:10 (0.0005 γ of ferritin nitrogen per 0.5 ml.).

Incubation mixture	Vasotropic activity		
	Original solution	Diluted 1:5	Diluted 1:10
Ferritin (control)	VDM	VDM	VDM
Liver slices + ferritin in O_2	"	Neutral	Neutral
Anaerobic liver slices + ferritin in O_2	"	VDM	VDM
" " " + Ringer-phosphate in O_2	" (mild)	Neutral	Neutral

injected intravenously into a group of rabbits in the form of an alum-precipitated suspension containing the equivalent of 0.15 mg. of ferritin nitrogen per ml. The suspension was injected on four consecutive days each week for 4 weeks. The material was administered in graduated doses as follows: 0.5 ml. for 2 days, 1.0 ml. for 2 days, 1.5 ml. for 4 days, 2.0 ml. for 4 days, and 3.0 ml. for 4 days. Serum was obtained 5 days after the last injection. Additional injections were given thereafter in order to maintain and increase the antibody titer of the serum. The quantitative precipitin reaction was performed (10) by the addition of varying amounts of the antigen solution to 1 ml. portions of the antiserum containing enough 0.9 per cent saline to produce a final volume of 3 ml. The mixtures were incubated at 37.5° for 30 minutes and then kept for 48 hours in the refrig-

[3] We are greatly indebted to Dr. E. A. Kabat, Neurological Institute, New York, for his advice and many details concerning the quantitative precipitin technique.

erator. The total nitrogen of the centrifuged and washed precipitates was determined by the micro-Kjeldahl method. Each supernatant was tested for the presence of excess antibody or antigen by the addition of either antigen or antiserum to aliquots. All analyses were performed in duplicate.

Rabbit antiserum to horse spleen ferritin, which yielded a precipitate with solutions of horse spleen ferritin, also gave precipitates when mixed with concentrated solutions of VDM (Fraction B, Table II) prepared from horse liver. The quantitative data are presented graphically in Fig. 3, which gives the values for the total nitrogen precipitated in the form of an
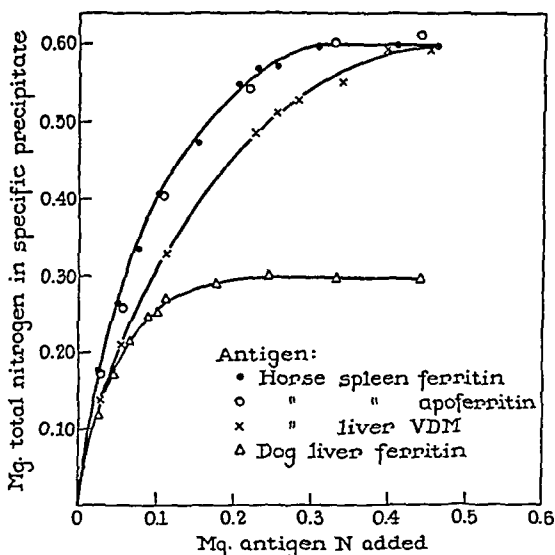
FIG. 3. Quantitative precipitin curves for rabbit antiserum to crystalline horse spleen ferritin.

antibody-antigen complex when solutions of ferritin or apoferritin were incubated with the antiserum to ferritin. The curves are identical, indicating that the antibody is directed towards the protein moiety of ferritin and is not influenced by the presence of the bound iron in high concentrations.

The curve (Fig. 3) shown for a preparation of horse liver VDM indicates that with equal quantities of ferritin nitrogen and of horse liver VDM nitrogen the total nitrogen in the precipitate obtained with ferritin was greater than that found with the VDM. As a consequence, the early portions of the curves are not superimposable. However, when increasing amounts of horse liver VDM were added, up to the maximal precipitating capacity of the antiserum, maximal precipitation of total nitrogen was

obtained at a level corresponding to that obtained with ferritin. Thus, the horse liver VDM solution contained ferritin and in addition some non-ferritin protein which was not precipitable by the antiserum. The quantitative data permit a calculation of the per cent ferritin nitrogen present in the horse liver VDM solution in relation to the total nitrogen content (11). Table VI gives a comparison of the relative amounts of ferritin and VDM nitrogen required to obtain identical amounts of total nitrogen in the precipitates. From this calculation, ferritin nitrogen comprised 65 per cent of the total nitrogen in the horse liver VDM preparation.

Fig. 3 also shows a cross-reaction between ferritin prepared from dog liver and horse spleen ferritin. Maximal precipitation occurred at a much lower level of total nitrogen in the specific precipitates than with horse

TABLE VI

Percentage Ferritin in Horse Liver VDM from Immunochemical Data (See Fig. 3)

The VDM activity of the horse spleen ferritin was obtained with 0.0005 γ of nitrogen per 0.5 ml., that for the horse liver VDM solution with 0.001 γ of nitrogen per 0.5 ml. The values for the total N in the specific precipitates were chosen so as to correspond to the portion of the curves where antibody is present in excess.

Total nitrogen in ppt.	Antigen N of ferritin	Antigen N of VDM	Per cent ferritin in VDM
mg.	*mg.*	*mg.*	
0.240	0.044	0.068	65
0.320	0.068	0.108	62
0.360	0.084	0.132	64
0.420	0.112	0.172	65
0.460	0.140	0.204	69
Average..			65

ferritin. These results indicate that the ferritins of these two species are immunologically related but not identical. However, identical curves have been obtained with horse ferritins from both the liver and spleen, thus establishing their immunological identity.

Comparable experiments were carried out with dog liver ferritin, a dog liver VDM preparation, and the antiserum obtained from rabbits immunized to crystalline dog liver ferritin. As with the horse spleen ferritin and horse liver VDM, the curve obtained with dog liver VDM was initially lower than that for the crystalline dog liver ferritin, but ultimately rose to a similar maximum. The antiserum to dog liver ferritin gave a cross-reaction with horse spleen ferritin. However, in this instance the curves were reversed, the lower maximum of total nitrogen in the precipitates being given by the horse ferritin. Rabbit antiserum to human liver ferritin gave a positive cross-reaction with horse spleen ferritin.

Identification of Ferritin with Naturally Occurring VDM by Immunological Procedures—The identity of hepatic VDM with ferritin did not preclude the possibility that the VDM activity present in the blood of dogs in irreversible shock might differ from ferritin. This might be approached by the direct isolation of ferritin from the plasma of dogs in shock. However, since the VDM activity in 0.5 ml. of such plasma would be expected to be equivalent to that given by 0.0005 γ of ferritin nitrogen, such an isolation was deemed impractical. Furthermore, addition of antiferritin serum to 0.0005 γ of ferritin nitrogen would not yield a visible precipitate for purposes of identification. For these reasons, the method of bioassay by the rat test was employed in conjunction with immunological procedures.

Preliminary to these experiments, ferritin was precipitated from a concentrated horse liver VDM solution by incubation for 30 minutes at 37.5° with an excess of antiserum to horse spleen ferritin. The precipitate was removed by centrifugation. The filtrate was assayed for VDM activity and found to be neutral. As a control, antiserum alone was tested and also found to be neutral. A similar incubation was then carried out with very low concentrations of horse liver VDM, comparable to that present in plasma from a dog in irreversible shock. No precipitate was obtained but the VDM activity was abolished. Hence, the antibody-antigen complex apparently did not dissociate sufficiently, following its injection, to elicit any vascular response in the rat test. Under similar conditions incubation of VDM with normal rabbit serum left the VDM activity unaffected.

VDM of Plasma from Dogs in Shock—Rabbit antiserum to crystalline dog liver ferritin was incubated with plasma from dogs in the hyporeactive phase of hemorrhagic shock. Under these experimental conditions, which are given in detail in Table VII, the VDM activity of the plasma was abolished. Normal rabbit serum was without effect. Antiserum alone exerted no vasotropic activity in the rat test. Inactivation of VDM in plasma from dogs in shock was likewise achieved by incubation with antiserum to horse spleen ferritin. This is further evidence of the immunological cross-reaction between horse and dog ferritins.

VDM from Anaerobic Liver Slices—VDM was prepared by a 2 hour anaerobic incubation of normal dog liver slices at 37.5° in 5 volumes of Ringer-phosphate solution. The VDM solution was then incubated with antiferritin serum as described above, with a resultant loss of VDM activity.

VDM of Plasma in Chronic Experimental Renal (Dog) and Essential (Human) Hypertension—In previous studies from this laboratory (12) it was observed that during the acute stage of experimental renal hypertension, induced in dogs by the application of the Goldblatt clamp, VEM appeared in the blood. However, after the establishment of a chronic hypertensive state, the blood gave a neutral test by the rat assay method.

This neutral test was found to result from the presence of high concentrations of both VDM and VEM in a ratio which led to mutual neutralization (13). This was demonstrated by the aerobic incubation of such "neutral" plasma with normal kidney slices, a procedure which inactivates VEM but not VDM. Following this inactivation of VEM, the dog plasma gave a strongly positive VDM reaction.

To complement this study, plasma samples from dogs with chronic renal hypertension, which gave "neutral" tests, were now incubated with antiserum to crystalline dog ferritin. Following incubation, these plasmas exerted pronounced VEM effects, indicating the unmasking of VEM through the formation of an inert VDM-antibody complex (Table VII).

TABLE VII

Inactivation of Naturally Occurring VDM by Antiferritin Serum

The VDM solution was tested for vasotropic activity after incubation of 2 ml. of the solution with 1.0 ml. of saline at 37.5° for 30 minutes. Another 2 ml. aliquot of the VDM solution was incubated in the same manner with 1.0 ml. of an antiserum to the appropriate ferritin (dog or human).

The hypertensive dog plasma was obtained from dogs made hypertensive by removal of one kidney and partial clamping of the renal artery of the remaining kidney. Hypertensive human plasma was obtained from patients with chronic essential hypertension.

Source of VDM	Vasotropic activity	
	Incubation with saline	Incubation with antiserum
Dog plasma, irreversible shock.....................	VDM	Neutral
" liver slices incubated in N_2....................	"	"
Hypertensive dog plasma........................	Neutral	VEM
" human plasma.....................	"	"

In cases of chronic essential hypertension in man, plasma was likewise found to give a "neutral" reaction which was converted to a strong VDM effect after aerobic incubation with normal kidney slices. The incubation of rabbit antiserum to crystalline human ferritin with plasma from such patients led to the appearance of a strong VEM reaction, indicating the removal of VDM. By way of control, similar studies were carried out with plasma from normotensive dogs and humans which are characterized by their neutral effect in the rat test. Neutral tests were also obtained after incubation of such plasmas with antiferritin serum.

DISCUSSION

Experiments have been described which led to the identification as ferritin of a hepatic vasodepressor, previously referred to as VDM. This

was accomplished by a combination of chemical and immunochemical procedures, together with the utilization of the rat mesoappendix test of Zweifach *et al.* (2).

In the course of this study, the question arose as to which portion of the ferritin molecule was responsible for its vasotropic effects. Apoferritin is a very homogeneous protein. Ferritin, on the other hand, is not a definite molecular species, but, as shown by the ultracentrifuge studies of Rothen (7), consists of a mixture of apoferritin-iron hydroxide (ferritin) and apoferritin. In the ferritin preparation studied by Rothen, apoferritin was present to the extent of 25 per cent. Results in our laboratory are confirmatory of Rothen's findings. Thus, we were able to separate chemically a crystalline ferritin preparation (No. 1162, Table IV) into a number of fractions in which the iron content varied from 0.93 to 2.20 mg. of iron per mg. of nitrogen. A similar fractionation of Preparation 1162 was accomplished by high speed centrifugation (Fractions 1162X and 1162Y, Table IV). When the various ferritin preparations with different iron to nitrogen ratios were compared with respect to VDM activity, the correlation of VDM activity with nitrogen content was excellent, whereas there was a poor correlation between activity and iron content. Indeed, on the basis of nitrogen content, apoferritin, which was devoid of iron, proved as active as ferritin. It is therefore the protein moiety of ferritin which is responsible for its vasodepressor activity.

The next problem which arose concerned the identity of the naturally occurring vasodepressor materials in the blood during the irreversible stage of hemorrhagic shock and in the chronic stage of experimental renal and human essential hypertension. The fractionation procedures which were evolved for the preparation of active VDM solutions were entirely dependent on the vascular changes observed in the rat test. Although this test could reveal a specific type of vasodepressor activity, there was no certainty that this vascular response was characteristic of only one substance in the body. Thus, the isolation of a single compound from the liver (ferritin) with VDM activity did not necessarily mean that the vasodepressor in the blood of dogs in irreversible shock was identical with or even related to it. Similar problems have arisen in the past with no direct solution; *e.g.*, the relation of the epinephrine-like substances in blood to epinephrine isolated from the adrenals.

Because of the protein nature of the ferritin-apoferritin complex, this uncertainty could be resolved by immunochemical methods. The specific combination of ferritin-antibody with ferritin in dilute solutions, together with the rat test for VDM activity, made it possible to determine whether any naturally occurring VDM was identical with ferritin. This was found to be the case for VDM in liver of dogs in irreversible shock, and for VDM in blood both during the irreversible stage of experimental

hemorrhagic shock and in the chronic stage of experimental renal and essential human hypertension. However, this procedure does not make it possible to determine whether it is ferritin or apoferritin which is responsible for VDM activity in these cases, because of the absence of a visible precipitate at such low concentrations of antigen. When larger amounts of these substances are present, as in hepatic concentrates, such a differentiation is possible, on the basis of the iron content of the specific precipitate formed when the antibody precipitates the ferritin from solution.

Of particular interest are the immunological cross-reactions between horse and human and between horse and dog ferritin. These cross-reactions have interesting physiological implications. Large quantities of antibody to horse, dog, rat, and human ferritins have been prepared and are being utilized for experiments on animals and man with a view to the elucidation and possible modification of the variety of conditions associated with derangements of the VDM-VEM mechanisms. These studies, which are being carried out with Dr. Zweifach and Dr. Baez of this laboratory, will be the subject of a separate report.

It is beyond the scope of this paper to discuss in detail the physiological rôle of ferritin in the regulation of the peripheral circulation. The participation of VDM and VEM in experimental shock, hypertension, and nutritional cirrhosis has been dealt with in other papers (1, 14, 15). On basis of these studies, it has been postulated that VDM and VEM are oppositely acting components of a homeostatic mechanism for the regulation of the peripheral circulation. In addition, it has been found that VDM exerts a profound antidiuretic effect (16) in the dog and rabbit by inducing an increased tubular resorption of water. This is a phenomenon of particular interest in relation to the antidiuresis which is observed in hepatic cirrhosis.

Hitherto, the only function of the ferritin-apoferritin system appeared to be that of iron transport and storage (17). To this may now be added the newly described and important function of participation in the regulation of the peripheral circulation. Further study will be required to establish the exact mechanism and significance of this function of ferritin.

SUMMARY

A hepatic vasodepressor principle (VDM) which has been found to participate in the regulation of the peripheral circulation has been con-

centrated from saline extracts of anaerobic beef, dog, horse, and human liver. The VDM activity of these concentrates, as measured by the effects induced on the terminal vascular bed, was proportional to their ferritin content. Crystalline ferritin and its iron-free component, apoferritin, were found to exert similar vascular effects.

By immunochemical procedures the VDM of hepatic concentrates was identified as ferritin. By similar procedures, naturally occurring VDM, present in the liver and blood of dogs in irreversible shock and in the blood during the chronic stage of experimental (dog) and essential (human) hypertension, was identified with ferritin or apoferritin.

These findings indicate that, in addition to iron transport and storage, the ferritin-apoferritin system plays an important rôle in the regulation of the peripheral circulation.

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DIMETHYLTHETIN AND DIMETHYL-β-PROPIOTHETIN IN METHIONINE SYNTHESIS*

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In a previous communication it was shown that choline and betaine are effective in promoting methionine synthesis from homocysteine in tissue homogenates (1). Data presented in this paper indicate that dimethylthetin, $(CH_3)_2^+ SCH_2COO^-$, which has been shown by Welch (2) to be lipotropic and has been reported by du Vigneaud (3) to promote growth on a methionine-free, homocysteine-containing diet, is 20 times as active as betaine in methionine formation. Dimethyl-β-propiothetin, $(CH_3)_2^+S(CH_2)_2COO^-$, recently isolated from *Polysiphonia fastigiata* by Challenger and Simpson (4) is also highly active. The enzyme for this transmethylation is found in the liver and kidney of all animals tested. Its high activity and general distribution suggest its biological importance in methionine synthesis.

Methods

Viobin extracts were prepared by stirring 5 gm. of Viobin[1] in 100 ml. of water for half an hour and filtering. Fresh extracts were prepared from organs of animals which had been killed by stunning and thoroughly bled. The organs were chilled, homogenized with 2 parts of buffer in the homogenizer of Potter and Elvehjem (5), and strained through cheese-cloth. The buffer (6) is composed of 0.0128 M sodium phosphate, pH 7.4, 0.123 M sodium chloride, 0.005 M potassium chloride, and 0.003 M magnesium sulfate.

Methylmercaptoacetic acid was prepared by Dr. M. Fling according to the method of Larsson (7).

Dimethylthetin was kindly given us by Dr. A. D. Welch.

Dimethyl-β-propiothetin was prepared by the method of Biilmann and Jensen (8).

DL-Homocystine was prepared from DL-methionine by the method of

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[1] Viobin preparations are commercially prepared desiccated and defatted raw tissues manufactured by the Viobin Corporation, Monticello, Illinois.

Butz and du Vigneaud (9) and reduced to DL-homocysteine as described by Riegel and du Vigneaud (10).

Dimethylglycine was prepared by the method of Schubert (11).

4 ml. of buffer solution containing the enzyme and substrates were incubated in 20 ml. beakers in an apparatus especially designed for non-manometric studies (12). In this apparatus as many as thirty 20 ml. beakers employed as reaction vessels are held in a stainless steel container fitted with a cover through which any gas mixture may be passed. The container is incubated at 38° and shaken at 90 oscillations per minute in a small water bath.

After incubation the solutions were deproteinized by the addition of 0.5 ml. of 30 per cent trichloroacetic acid and 1 ml. of water to each beaker. With guinea pig liver homogenates after the addition of trichloroacetic acid, the solutions were brought to a boil in the incubation apparatus in order to get filtrates which would remain clear in the analytical procedure. Filtrates of other organs offered no difficulty.

Methionine was determined by a modification of the method of McCarthy and Sullivan (13). The procedure was as follows: To 2 ml. of the trichloroacetic acid filtrate were added 0.2 ml. of 5 N NaOH followed by 0.1 ml. of 1 per cent freshly made sodium nitroprusside. The solutions were incubated at 38° for 5 to 10 minutes and then 1 ml. of an acid mixture consisting of 9 volumes of concentrated hydrochloric acid and 1 volume of 85 per cent phosphoric acid was added. After 10 minutes the solutions were read in a Klett-Summerson colorimeter with a green filter.

If the solutions are cooled in ice before the addition of strong acid, homocysteine reduces the color by an amount which increases with increasing concentration of methionine. When this cooling step is omitted as described, the decrease in color due to homocysteine is a small and constant value over a wide range of methionine concentrations, and no difficulty is encountered with gas bubbles during the measurement of the color. 1 mole of methylmercaptoacetic acid formed by the demethylation of dimethylthetin gives a color equivalent to 0.6 mole of methionine in this determination. Accordingly, all apparent increases of methionine due to the addition of dimethylthetin must be divided by 1.6 to compensate for the equivalent amount of methylmercaptoacetic acid formed in the reaction. Dimethyl-β-propiothetin is chromogenic, but it can be destroyed by allowing the solutions to stand overnight after the addition of 5 N NaOH. The nitroprusside is added the following day and the determination continued as described. The chromogenic power of methylmercaptopropionic acid, the demethylated product of dimethyl-β-propiothetin, is approximately 0.9 that of methionine on a mole basis.

Results

In rat liver choline, betaine, dimethyl-β-propiothetin, and dimethylthetin show significant activity in methionine formation (Table I). Dimethylethanolamine, dimethylglycine, and methylmercaptoacetic acid, the compounds formed by the removal of one methyl group from choline, betaine, and the dimethylthetin, respectively, are inactive.

If homocysteine is present in excess, it can be directly shown that only one methyl group is transferred per mole of dimethylthetin (Fig. 1). The reaction with betaine is too slow to reach equilibrium; only about 0.6 mole equivalent of methyl is transferred per mole of betaine in 24 hours.

TABLE I

Methionine Formation in Rat Liver Homogenate

1 ml. of 1:4 homogenate. Homocysteine 25 mg. per cent; all other substrates 12.5 mg. per cent. Total volume 4 ml.; gas phase, nitrogen; time, 3 hours; temperature, 38°. Methionine values are the average of three determinations.

Reaction mixture	Methionine found
	mg. per cent
Homocysteine..	1.0 ± 0.05
" + choline...................................	2.6 ± 0.05
" + betaine...................................	4.2 ± 0.01
" + dimethyl-β-propiothetin..................	4.5* ± 0.1
" + dimethylthetin............................	8.4* ± 0.05
" + methylmercaptoacetic acid..................	1.0 ± 0.0
" + dimethylglycine...........................	1.0 ± 0.1
" + dimethylethanolamine......................	1.1 ± 0.05
" + monomethylethanolamine....................	1.3 ± 0.2

* Corrected for chromogenic value of demethylated product as indicated in section on methods.

Table II shows the distribution of the betaine- and dimethylthetin-transmethylating enzymes and approximate $Q_{methionine}$ values for various tissues. Only liver and kidney show activity with betaine and dimethylthetin.

The effect of pH on the activity of the dimethylthetin enzyme is shown in Fig. 2. The optimum pH is about 7.8.

Neither the betaine nor the dimethylthetin enzyme is inactivated by simple dialysis.

The formation of methionine from dimethylthetin, dimethyl-β-propiothetin, betaine, or choline was not inhibited by cyanide, azide, arsenate, or arsenite.

The two enzymes can be distinguished by the greater stability of the dimethylthetin enzyme at pH 4.0 as shown in Fig. 3. The variation of the
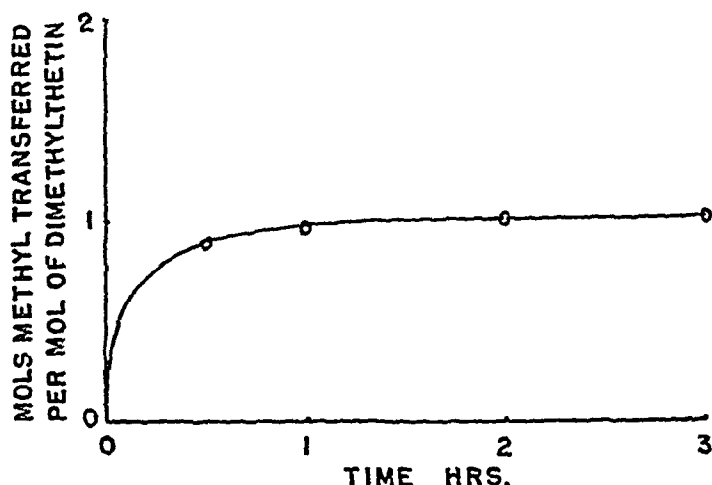
FIG. 1. Number of methyl groups transferred per mole of dimethylthetin. 1 ml. of 5 per cent solution of Viobin liver in buffer (6); dimethylthetin 5 mg. per cent; L-homocysteine 12.5 mg. per cent. Temperature, 38°; gas phase, nitrogen.

TABLE II

Distribution and Activity of Methionine-Forming Enzyme Systems

The figures are $Q_{methionine}$; average of two determinations; probable error ±5 per cent.

Each vessel contained 1 ml. of homogenized guinea pig or rat tissue diluted 1:4, or 1 ml. of a 5 per cent Viobin solution. DL-Homocysteine 25 mg. per cent, betaine or dimethylthetin 12.5 mg. per cent. Total volume, 4 ml.; gas phase, nitrogen; time, 3 hours; temperature, 38°.

	Guinea pig			Rat			Hog (Viobin)		
	Dimeth-ylthetin	Betaine	$\dfrac{\text{Dimethyl-thetin}}{\text{Betaine}}$	Dimeth-ylthetin	Betaine				$\dfrac{\text{Dimethyl-thetin}}{\text{Betaine}}$
Liver.............	1.5	0.09	17	1.5	0.11	14	1.3	0.14	9
Kidney..........	0.3	0.06	5	0.2	0.03	7	0.9	0.03	30
Spleen...........							0	0	.
Muscle..........	0	0							
Pancreas........							0	0	

ratio of $Q_{dimethylthetin}$ to $Q_{betaine}$ (Table II) also points to two *different* enzymes for the two methylators.

The dimethylthetin enzyme may be purified free of betaine enzyme and concentrated about 100-fold by precipitating a 5 per cent aqueous solution

of Viobin[1] liver with alcohol. The fraction precipitated at between 11.5 and 18 per cent alcohol contains almost all the activity of the whole extract. While the crude homogenate is effective with homocystine and
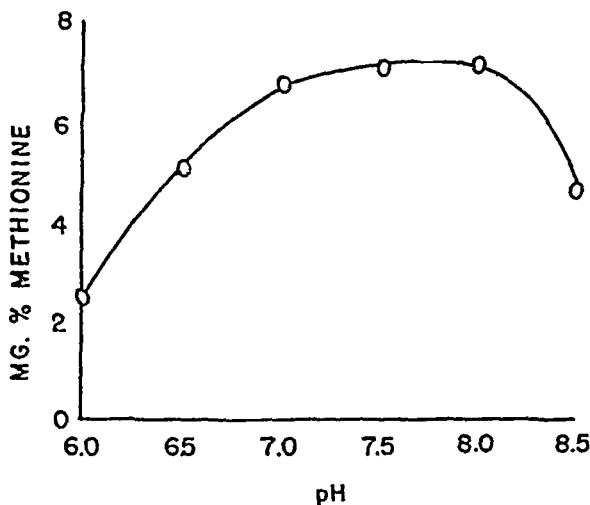
Fig. 2. Effect of pH on methionine formation from dimethylthetin. 1 ml. of 5 per cent Viobin; dimethylthetin 12.5 mg. per cent, DL-homocysteine 12.5 mg. per cent. Temperature 38°; gas phase, nitrogen; time, 1 hour.
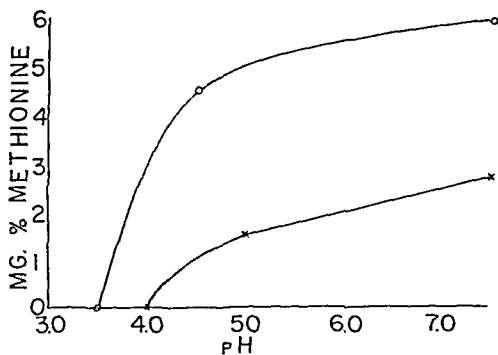
Fig. 3. Stability of enzyme to pH. Upper curve, dimethylthetin transmethylase; lower curve, betaine transmethylase. 5 per cent Viobin allowed to stand 24 hours at 5° at given pH, and relative activity at pH 7.5 determined at 38°. Substrates 12.5 mg. per cent. Gas phase, nitrogen; total volume, 4 ml.; time, 3 hours.

homocysteine, this fraction reacts only with homocysteine. Although the dimethylthetin transmethylase can be prepared free from the betaine transmethylase by further alcohol fractionation, all active betaine transmethylase preparations have had dimethylthetin transmethylase activity.

Whether the latter enzyme is required for betaine activity is still uncertain.

An attempt was made to demonstrate the presence of dimethylthetin in animal tissues. Neutral and acidified aqueous and alcoholic extracts of Viobin[1] pancreas, spleen, kidney, and liver, fresh beef pancreas, and guinea pig and rat liver and kidney were inactive with a partially purified dimethylthetin enzyme. Liver and kidney homogenates of the rat and guinea pig were allowed to stand at pH 4.0 at 5° for 18 hours to destroy all but the dimethylthetin methylating system. Any increase in methionine on addition of homocysteine in such a system could be considered as evidence of preformed dimethylthetin. No increase in methionine occurred. These experiments seem to exclude any significant quantity of preformed dimethylthetin in these tissues. On the addition of labile methyl donors to homogenates there is a slight synthesis of methionine in most experiments, suggesting the presence of small amounts of preformed homocysteine.

None of these reactions is reversible under our conditions; i.e., methionine will not remethylate dimethylglycine, dimethylethanolamine, or mercaptoacetic acid aerobically or anaerobically in the presence of high energy-yielding metabolites. These reactions were studied by measuring the change in methionine concentration in the presence of these putative methyl acceptors.

Although dimethylthetin is very effective with homocysteine, it will not methylate glycocyamine in rat or guinea pig liver homogenates.

DISCUSSION

Evidence has been presented that there are at least four compounds which can furnish methyl groups for methionine synthesis in tissue homogenates. These compounds, dimethylthetin, dimethyl-β-propiothetin, betaine, choline, are all "onium" compounds characterized by the coordination of an additional methyl group to sulfur or nitrogen, and they all react in the absence of oxygen or energy donors. It has been directly demonstrated in these and previous studies (1) that the methyl groups in dimethylglycine, dimethylethanolamine, and methylmercaptoacetic acid are not transferred under conditions in which a methyl of the "onium" compound is labile. This confirms the findings in feeding experiments on the availability of methyl in dimethylglycine and dimethylaminoethanol (14).

The methyl of methionine, which is held by a covalent bond to sulfur, but is nevertheless labile, requires energy for its transfer to glycocyamine (15) and to nicotinamide (16).

Du Vigneaud and his collaborators have proved rigorously that the methyl groups of choline, betaine, and methionine constitute a dietary

"pool" of physiologically interchangeable methyl groups. The evidence reported here indicates that in the tissues there does not exist a "pool" of labile methyl groups in the sense that the transfer is directly between any two members of the dietary pool of labile methyl compounds. It seems likely that rather than a "pool" there are specific methyl donors for each methyl acceptor (*i.e.*, the methylation of glycocyamine by methionine (15)), and that a given methyl compound may be related to another only indirectly through a series of methyl transfer reactions.[2]

We have so far failed to find any methyl transfer reaction which is reversible in the usual chemical sense. Methionine, for example, does not directly remethylate dimethylethanolamine, dimethylglycine, or methylmercaptoacetic acid.

The present findings indicate that the physiological transfer from methionine to choline must be a cyclic process in which some and possibly all steps are irreversible. In some stages of the cycle oxidation and presumably, therefore, energy is required; other stages may proceed anaerobically. In such a dynamic state a given labile methyl-containing compound cannot be utilized or depleted to the same extent by all methyl acceptors. This is in accord with the fact that not all methyl donors are equally effective in overcoming growth inhibition by such compounds as glycocyamine (17, 18) and nicotinamide (19, 20).

The effectiveness of dimethylthetin suggests its importance in the biological synthesis of methionine. Its apparent absence from animal tissues may be due to its rapid demethylation in the presence of homocysteine, or it may be present and function in only catalytic amounts. The occurrence of dimethylthetin in the dietary sources has not been studied. Its homologue, dimethyl-β-propiothetin, has been isolated from algae (4) and may be present in pineapple (21). This compound may, therefore, prove to be more important biologically than dimethylthetin despite its lower activity in methionine synthesis.

SUMMARY

1. An enzyme has been isolated in a partially purified state which transfers a methyl group from either dimethylthetin or dimethyl-β-propiothetin to homocysteine.

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[2] The data presented here do not establish a direct methyl transfer from all four compounds to homocysteine. Unpublished evidence suggests that choline is first oxidized to betaine. Whether the latter compound transfers directly or through a methylthetin derivative has not yet been determined.

2. Dimethylthetin is 10 to 20 times as effective as betaine as a methyl donor in tissue homogenates. Dimethyl-β-propiothetin is more effective than betaine.

3. The enzyme is found in the liver and kidney of rat, guinea pig, and hog, but is absent from muscle, pancreas, and spleen.

4. The reaction proceeds until one methyl group has been transferred from dimethylthetin to homocysteine. Mercaptoacetic acid is inactive.

5. Homocystine is completely inactive as a methyl acceptor in purified extracts.

6. The reaction is independent of O_2 and is not inhibited by oxidative poisons.

7. The dimethylthetin transmethylase is distinguished from the betaine transmethylase by its stability at pH 4.5.

8. The possible rôle of dimethylthetin and dimethyl-β-propiothetin in the biological formation of methionine is discussed.

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BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

X. FRACTIONATION STUDIES OF NORMAL AND IMMUNE HORSE SERUM*

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A large portion of the antibody proteins of horse serum resides in a fraction possessing an electrophoretic mobility between the normal serum γ-globulins and the lipide-rich β-globulins (1-6). While this component has been designated β-globulin by Kekwick and Record (3) and has been shown to consist of two components (β_1 and β_2), it is analogous to the T component of van der Scheer and Wyckoff (4). We have described the corresponding antibody-rich protein fraction from normal human plasma as γ_1-globulin to distinguish it from the normal serum γ_2-globulin (7). We shall retain this terminology. The need for the separation of these two globulins in order that both their biological and physicochemical properties may be elucidated is evident.

Recently Smith and Gerlough (8) applied the low temperature ethanol fractionation procedures of Cohn et al. (9) developed for the fractionation of human plasma to the separation of the tetanus antitoxin from the plasma of hyperimmunized horses. They found the antitoxic activity to be associated with various fractions and concluded that the pepsin digestion methods of antibody recovery (10, 11) were more suitable for the concentration of such immune plasma systems than the ethanol type of fractionation. Other work on the ethanol fractionation of various animal sera (12-15) has indicated that the successful separation of any electrophoretically well defined protein entity from a given animal serum will require specific conditions and that the methods designed for human plasma cannot be applied to other animal plasmas *in toto*.

We have found it possible to develop conditions whereby the antibody content of the serum of hyperimmunized horses may be separated in high yield by the low temperature ethanol method in a single precipitation step. This fraction may, however, be divided into various electrophoretic components by subsequent refractionations. The methods of obtaining such

fractions from normal and hyperimmunized horses and a description of certain of their biological and physicochemical properties form the basis of this report.

EXPERIMENTAL

Plasma or serum of normal and hyperimmunized horses was used as the source material.[1] The immune serum samples were usually aliquots of relatively large pools of antiserum to either tetanus or diphtheria antitoxin. In addition a pooled serum sample of two horses that had each been immunized simultaneously with diphtheria, tetanus, and a heterologous gas gangrene (vibrion septique and *Bacillus welchii*) toxoid, and *Hemophilus pertussis* and formalized pneumococcus type III vaccines was also studied. While it was realized that type III pneumococcus vaccine is a poor antigen, it was used because the type-specific polysaccharide is nitrogen-free and allows for the ready determination, by quantitative precipitation, of the antibody produced. Plasma samples were defibrinated by the addition of sufficient calcium ion to permit clotting, followed by stirring to remove the fibrin formed. These sera were then fractionated by means of the aqueous ethanol precipitation techniques and as usual temperature, pH, protein concentration, alcohol concentration, and ionic strength were carefully controlled. The fractionation experiments were evaluated in terms of electrophoretic composition and of protein and antibody yields resulting from controlled variations of the several variables of fractionation. All electrophoretic experiments at pH 8.6 were carried out in veronal buffer of ionic strength 0.1 for 9000 seconds at a constant potential gradient of 6.0 to 6.5 volts per cm. The mobility experiments were performed in solutions of ionic strength 0.1 in which sodium chloride made up 80 per cent of the ionic strength, the remainder being the contribution of a univalent buffer salt. Velocity sedimentation analyses were carried out with 0.7 per cent protein solutions in the oil turbine ultracentrifuge at 220,000 times gravity, a schlieren optical method being used to record the position of the moving boundaries as a function of time.[2]

The antibody assays were obtained by the following tests.[3] Preliminary diphtheria antitoxin titers were obtained by Ramon flocculation and final values by guinea pig intracutaneous (L+) tests. The antibodies to *Bacillus tetanus*, vibrion septique, and *Bacillus welchii* toxoids were assayed by the standard mouse tests. The antibody to *Hemophilus pertussis*

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[1] All of the horse serum samples were supplied through the courtesy of Eli Lilly and Company.

[2] The sedimentation velocity experiments were performed by Mr. E. M. Hanson.

[3] These assays were carried out in the laboratories of Eli Lilly and Company, Indianapolis, Indiana.

was determined by agglutination procedures. Pneumococcus antibody assays were attempted by agglutination, by the capsular swelling or *Quellung* reaction, and by precipitin tests with the specific polysaccharide.

Fractionation Results

Preliminary experiments indicated that the electrophoretically heterogeneous γ-globulins and associated antibodies of horse serum could be removed almost quantitatively by precipitation with 25 per cent ethanol at pH 7.5 to 7.8. In addition to the γ-globulins these initial precipitates contained from 5 to 15 per cent of β-globulins. Subsequent experiments were carried out to remove these β-globulins and to provide a γ-globulin fraction made up of proteins having two electrophoretic constituents, the one described as γ₁-globulin and the other as γ₂-globulin. The fractionation conditions which evolved for this purpose are shown in the accompanying Diagram 1.

<div align="center">DIAGRAM 1</div>

1000 ml. serum, 3000 ml. H_2O; adjust to pH 7.7 (±0.1); add 50% EtOH to 25%; temperature −6° to −7°; centrifuge

Ppt. A; largely γ-globulins with some β-globulins   Supernatant I
Suspend (dried protein or paste) in sufficient
  H_2O to give 1% solution; 0.05 м acetic acid
  added to pH 5.2 to 5.8; ethanol concentration
  0 to 10%; centrifuge at 0° to −2°

Ppt. A-A; largely β-globulins   Supernatant II; 0.5 м
     NaHCO₃ added to pH
     7.2 to 7.4; 50% ethanol
     added to 25%; centrifuge at −6° to −7°

Ppt. A-B γ-globulins   Supernatant III; discard

The electrophoretic diagrams of a typical series of fractions employing antidiphtheritic horse serum as starting material are shown in Fig. 1. From 80 to 100 per cent of the serum antibodies to diphtheria and tetanus toxins are usually found in Precipitate A. Considerable care must be taken to keep all precipitates as cold as possible during their removal and suspension prior to lyophilization to prevent marked destruction of antibody. Occasionally low yields (50 to 60 per cent) are experienced in the initial precipitate but, since the antibody is not found in the supernatant it appears that such marked losses are due to an improper handling of Precipitate

A, resulting in antibody destruction.   In Table I are shown the results of various typical fractionations on diphtheria and tetanus antisera in terms of yields of protein and antibody.   The antibody recovery data must
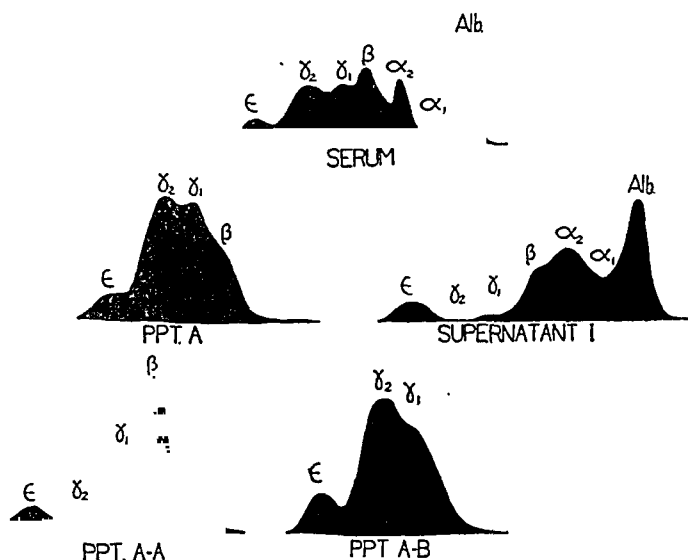
FIG. 1. Descending electrophoretic patterns of a hyperimmunized horse serum and fractions.

TABLE I

Antibody and Protein Recoveries from Serum of Hyperimmunized Horses

Antisera to	Units of antibody per 100 ml. plasma*	Weight of ppt. per 100 ml. serum			Units of antibody recovered per 100 ml. serum		
		Ppt. A	Super-natant to Ppt. A	Ppt. A-B	Ppt. A	Super-natant to Ppt. A	Ppt. A-B
Tetanus toxoid	20,000	5.07		3.22			24,200
Diphtheria toxoid	45,000	5.14		3.35			46,900
" "	129,000	5.24	3.17	3.51	98,000	3170	70,200
" "	65,000	5.55	3.36	3.47	55,500	2520	36,800
" "	57,500	5.21			52,100		.
Tetanus toxoid	45,000	4.10			24,600		

* Plasma diluted with anticoagulant.

be considered in relation to the shortcomings of the assay procedures. The data indicate that the major portion of the antibody is recovered in Precipitate A.

Refraction of this precipitate to give Precipitate A-B may be ac-

companied by excellent yields in some cases and by considerable losses of antibody in others. It appears again that losses of antibody at this point may be due to causes just mentioned. Approximately 10 gm. of protein for every 50 gm. of Precipitate A are lost in preparing Precipitates A-A and A-B under the conditions employed for their separation. It can be seen from Table I that the major portions of the antibody are recovered in Precipitate A or in its subfraction Precipitate A-B. Approximately 2 to 5 per cent of the initial serum antibody may be recovered by lyophilization

DIAGRAM 2

50 gm. Ppt. A, suspended in 4000 ml. H_2O; 0.05 M HAc added to pH 5.38; diluted to 5000 ml.; $\mu = 0.0014$; centrifuged at 0°

7.8 gm. (dry) Ppt. A-A

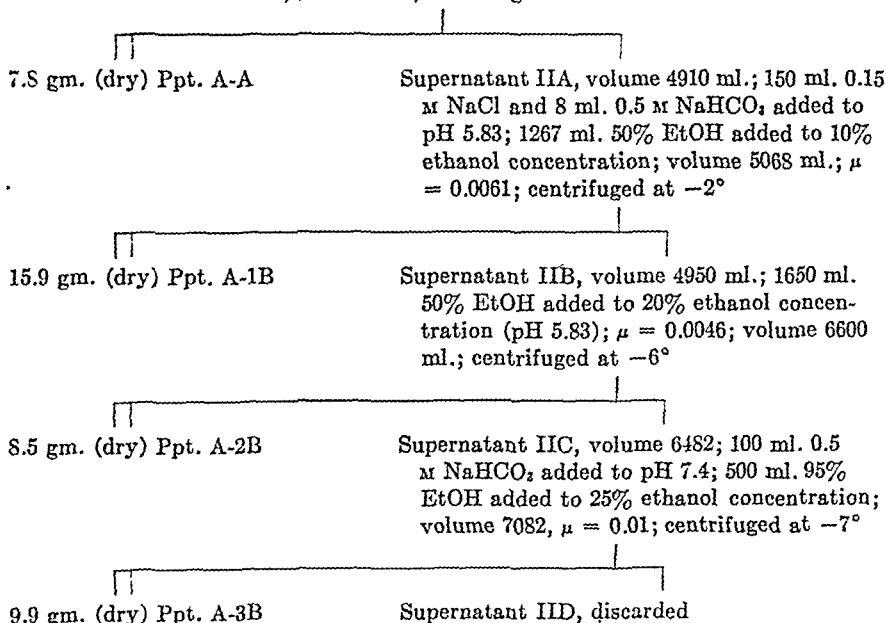Supernatant IIA, volume 4910 ml.; 150 ml. 0.15 M NaCl and 8 ml. 0.5 M NaHCO$_3$ added to pH 5.83; 1267 ml. 50% EtOH added to 10% ethanol concentration; volume 5068 ml.; $\mu = 0.0061$; centrifuged at −2°

15.9 gm. (dry) Ppt. A-1B

Supernatant IIB, volume 4950 ml.; 1650 ml. 50% EtOH added to 20% ethanol concentration (pH 5.83); $\mu = 0.0046$; volume 6600 ml.; centrifuged at −6°

8.5 gm. (dry) Ppt. A-2B

Supernatant IIC, volume 6482; 100 ml. 0.5 M NaHCO$_2$ added to pH 7.4; 500 ml. 95% EtOH added to 25% ethanol concentration; volume 7082, $\mu = 0.01$; centrifuged at −7°

9.9 gm. (dry) Ppt. A-3B

Supernatant IID, discarded

of Supernatant I. The yield of antibody into Precipitate A-A is relatively low. In a typical experiment, Precipitate A-A showed 100 units of tetanus antitoxin per gm. while Precipitate A-B gave 8000 units per gm. Subfractionations of Precipitate A-A give products with very low antibody content. They are composed largely of proteins moving with an electrophoretic mobility of -4.0×10^{-5} sq. cm. per volt per second (pH 8.6). Such findings indicate that the antitetanus activity of horse serum proteins does not ordinarily extend into the electrophoretic region of the horse serum designated as β-globulin in Fig. 1.

The sera of the two horses immunized to a series of antigens were pool

and fractionated to yield the usual initial antibody-rich Precipitate A. These precipitates were subfractionated and the distribution of the antibodies in the various fractions was studied.   The subfractionation conditions are shown in Diagram 2.

The predominant feature of this subfractionation was the separation of the usual Precipitate A-B into three fractions, a γ_1-globulin, a γ_2-globulin, and a mixture of these two globulins.

In Fig. 2 are shown the electrophoretic compositions of these subfractions.   It is readily apparent that the component labeled γ_1-globulin is
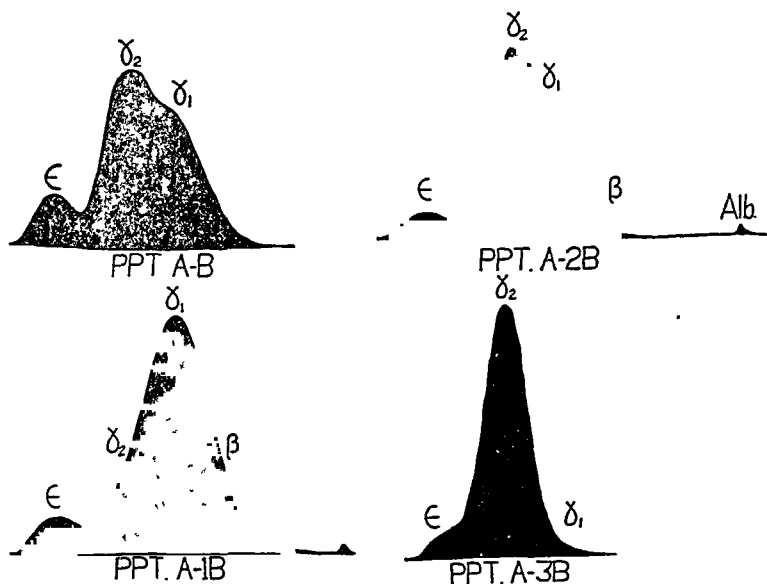
FIG. 2. Descending electrophoretic patterns of Precipitate A-B of a hyperimmunized horse serum and its subfractions.

relatively heterogeneous electrically and the fraction as a whole has a considerably higher electrophoretic mobility than does the γ_2-fraction.

Antibody and protein yield data are shown in Table II.   As was anticipated, no antibodies to pneumococcus type III organisms were found in Precipitate A as tested by agglutination, capsular swelling phenomena, or by precipitin reactions with pneumococcus type III polysaccharide.   The horses evidently failed to produce antibodies to the formalized pneumococcus vaccine which was employed.   Antibody production to vibrion septique toxoid was relatively low (less than 200 units per gm. of Precipitate A) and the subfractions were not assayed further.   The remaining antibodies were present in relatively low titer but were of sufficient magnitude

to make possible a study of their distribution into the various fractions. Practically no antibody was found in the supernatant to Precipitate A. The subfractions of Precipitate A showed varying titers of the several antibodies studied. The initial precipitate removed at pH 5.2 and low ionic strength (Precipitate A-A) contained very small amounts of antitoxin but contained as much, or more, agglutinin for *Hemophilus pertussis* on a weight basis as did the parent fraction (Precipitate A). Approximately 10 to 15 per cent of the diphtheria and tetanus antitoxins was found in Precipitate A-1B with the remainder appearing in the more soluble protein (pseudoglobulin in nature) making up Precipitates A-2B and A-3B. The diphtheria antitoxin showed a relatively higher titer in the γ_2-globulin fraction (Precipitate A-3B) than was true for tetanus antitoxin. The antitoxin to *Bacillus welchii* was found to be rather well dis-

TABLE II

Antibody Content of Subfractions of Antibody Fraction of Hyperimmune Horse Serum

| Weight of fraction | Fraction | Units antitoxin per gm. protein | | | Hemophilus pertussis agglutinin titer |
		Diphtheria	Tetanus	Bacillus welchii	
gm.					
50	A*	3000	1000	300	1-1280
7.8	A-A	300	100	−25	1-2560
15.6	A-1B (γ_1)	1000	500	300	1-160
8.4	A-2B ($\gamma_1 + \gamma_2$)	5000	4000	400	1-160
9.8	A-3B (γ_2)	3000	1000	500	1-1280

* The supernatant to Precipitate A contained less than 2 per cent of the antibody activity of this fraction.

tributed in all of these fractions. The *Hemophilus pertussis* agglutinins showed a high concentration in the γ_2-globulin fraction as compared to the γ_1 and the $\gamma_1+\gamma_2$-globulin subfractions. Such a finding might have been predicted from the electrophoretic studies of van der Scheer *et al.* (5). The high titer of this antibody in the euglobulin type of precipitate (Precipitate A-A) is, however, quite surprising in view of the small amount of γ_2-globulin in this fraction and is in contrast to the antitoxin distribution in the same fraction.

Quantitative Diphtheria Antitoxin Assays—A γ_1-globulin, a γ_2-globulin, and a mixture of them (analogous in electrophoretic composition to Precipitates A-1B, A-3B, and A-2B respectively of Fig. 2) were prepared from pooled antidiphtheritic horse serum that assayed approximately 800 units per ml. The γ_1-globulin preparation, however, was separated from a $\gamma_1+\gamma_2$-globulin mixture (Precipitate A-2B), since this fraction has a relatively higher antidiphtheritic titer in contrast to Precipitate A-1B (see

Table II). No attempt was made to recover large amounts of antibody, attention being focused on the recovery of electrophoretically well defined fractions. The $\gamma_1+\gamma_2$-globulin fraction contained approximately equal amounts of the two component proteins. The antibody contents of these preparations were determined by the quantitative methods as elaborated by Heidelberger and associates (16).[4] These results, summarized in Table III, show a diphtheritic antitoxin distribution in the various fractions that was analogous to that found by *in vivo* assay in similar fractions of the polyvalent horse serum (see Table II). The shape of the quantitative precipitin curves for these fractions was essentially the same as that obtained by Kabat (17) in plotting the Pappenheimer and Robinson (18) data for the diphtheria toxin-antitoxin (horse) reaction. In agreement with results of Kekwick and Record (3) the γ_2-globulins were found to flocculate more readily with toxin than the γ_1-globulins.

TABLE III

Diphtheria Antitoxin Content of Various γ-Globulin Fractions by Quantitative Precipitin Methods

Fraction	Units of diphtheria antitoxin per gm. protein	Per cent antitoxin of fraction
γ_1-Globulins	9000	10.4
$\gamma_1+\gamma_2$-Globulins	6750	7.8
γ_2-Globulins	3070	3.6

Fractionation of Serum during Course of Immunization—Changes in the plasma proteins from a single horse were studied during the course of immunization to diphtheria toxoid. Serum samples were collected before and at various times during the immunization period. Unfortunately the animal employed did not develop antitoxin above 400 units per ml. and the experiment was discontinued at this point. The serum fractions obtained do, however, show the shift toward the development of large amounts of γ_1-globulin, as was expected from the previous serum electrophoretic studies of van der Scheer *et al.* (5, 6) and Kekwick and Record (3). Electrophoretic patterns and yields of some of the serum samples fractionated are shown in Fig. 3. A marked increase in the yield of Precipitate A-B as immunization continued is readily apparent. The predominating feature is the gradual increase of the γ_1-globulin. The effect is most readily observed as the electrophoretic diagrams for Precipitates A-3B are studied. As the γ_1-globulin content of the serum increases it likewise becomes

more difficult to prepare a γ_2-globulin fraction (Precipitate A-3B) which does not show the presence of considerable γ_1-globulin. As previously noted by Kekwick and Record (3), there was an increased production of the γ_2-globulin during the initial stage of immunization. Thus, by the 7th day of immunization this component showed a marked enhancement without any increase in the amount of γ_1-globulin, but as immunization progressed the level of the γ_1-globulin rose.
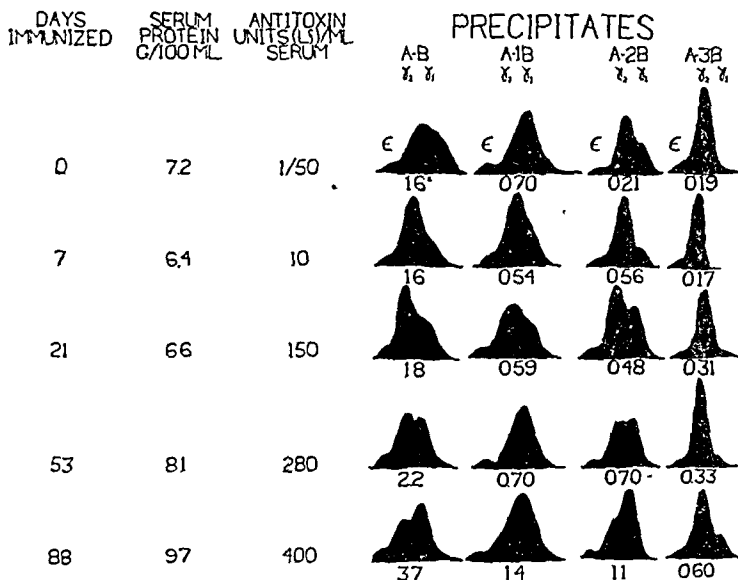
FIG. 3. Descending electrophoretic patterns and yield data of serum fractions from a single horse during immunization to diphtheria toxoid. The numbers under patterns are the yields of precipitate in gm. for 100 ml. of serum.

Fractionation of Normal Horse Sera—Serum samples which had been taken from nine normal horses were analyzed electrophoretically and fractionated individually in order to study the variation in composition and yield that one might expect when working with the plasma from single animals. The yield data of the fractions obtained from the sera examined are shown in Table IV. It is apparent that a great deal of individual variation may be expected among so called "normal" animals. Another feature of these and previous experiments was the increased difficulty in removing the β-globulins associated with Precipitate A into Precipitate A-A. This is in rather marked contrast with the results obtained when serum from hyperimmunized animals is used.

Electrophoretic Mobility Studies—The electrophoretic mobilities of γ_2-

and γ_1-globulin preparations obtained from antidiphtheritic plasma were determined for a series of pH values. The γ_1-globulin fraction represents that portion of Precipitate A from an antidiphtheritic serum which was soluble at pH 5.2, ethanol 10 per cent and ionic strength of 0.002, but which was insoluble at pH 6.1. This globulin preparation is somewhat analogous to Precipitate A-1B of Fig. 2, except that it was far more homogeneous electrically than the usual Precipitate A-1B.. The γ_2-globulin was a frac-

TABLE IV

Fractionation Results for Normal Horse Serum

Material		Per cent electrophoretic composition						Protein, gm. per 100 ml. starting serum
		γ_2	γ_1	β	α_2	α_1	Albumin	
Serum	Range	18–31	9–17	11–24	10–22	7–13	21–29	6.4–8.7
	Average	22	14	17	14	9	24	7.6
Ppt. A	Range	29–46	25–31	15–35	4–11	0–3	1–4	2.7–5.6
	Average	40	28	22	8	1	2	4.1
" A-A	Range	6–9	23–36	40–57	11–22	0–5	0–2	0.2–1.1
	Average	8	28	46	12	3	1	0.7
" A-B	Range	53–73	21–32	7–15	0–3	0	0–1	1.3–3.5
	Average	60	25	12	1	0	1	2.5
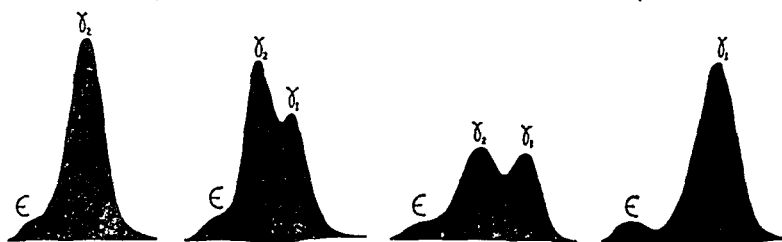
FIG. 4. Descending electrophoretic patterns of γ_1- and γ_2-globulins and mixtures of these two proteins.

tion analogous to Precipitate A-3B of Fig. 2. The electrophoretic patterns of these fractions in pH 8.6 veronal buffer of ionic strength 0.1 are shown as γ_1- and γ_2-globulins in Fig. 4. It can be seen that the γ_1-globulin has a slightly asymmetric pattern. The marked electrical inhomogeneity in this fraction has been already mentioned and, even though the γ_2-globulin preparation appeared to be more homogeneous from its electrophoretic diagram, the heterogeneity constant (19) of various preparations gave values in the neighborhood of 1×10^{-5} sq. cm. per volt per second.[5] The

[5] Anderson, E. A., and Nichol, J. C., unpublished experiments.

mobility *versus* pH values for the two protein fractions are plotted in Fig. 5. The average isoelectric points of the γ_1- and the γ_2-globulin components in buffer solutions of 0.1 ionic strength are 5.6 and 7.6 respectively. With the exception of the γ_1-globulin fraction at pH 8.6 these proteins gave single, apparently symmetrical peaks over the entire pH range studied.

Our investigations indicate that these globulin fractions are merely proteins of closely related isoelectric point and mobility. Dependent upon the conditions of separation there may be obtained arbitrary fractions which contain antibody and which have electrophoretic mobilities from -1.0 to -3.5×10^{-5} sq. cm. per volt per second in buffer of pH 8.6 and ionic strength 0.1. Hence the γ_1- and γ_2-globulin fractions employed for the
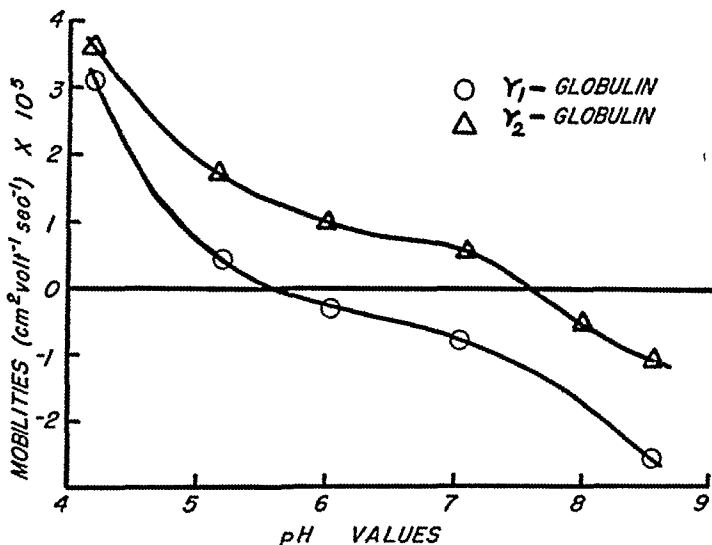
FIG. 5. pH mobility curve of a γ_1-globulin and a γ_2-globulin fraction

determination of isoelectric point are merely two fractions, the molecules in each being more closely related electrically than are those of the parent fraction (Precipitate A-B, Fig. 1). In addition to the patterns of the relatively homogeneous fractions, Fig. 4 also shows the diagrams for two mixtures of γ_1- and γ_2-globulins. It is readily apparent that a series of protein fractions showing variations in the average net charge at a given pH may be separated from horse serum. This fact is substantiated by the electrophoretic diagrams and analytical data for the hyperimmune horse serum antibody fractions of Smith and Gerlough (8).

Sedimentation Studies—Various preparations of γ_2- and of γ_1-globulins and mixtures thereof were studied in the Svedberg high velocity oil turbine ultracentrifuge. Approximately 80 per cent of these globulin fractions con-

sisted of material having a sedimentation constant (s_{20w}) of 6.8 or 7.2 Svedberg units, respectively.   The remaining 20 per cent of the protein in each case sedimented with velocities between 8 and 15 Svedberg units and exhibited no well defined molecular components in this range.   Some schlieren patterns of these proteins obtained during velocity sedimentation experiments are shown in Fig. 6.   The main component of both of these fractions was molecularly monodisperse, showing no increase in the apparent diffusion constant as sedimentation progressed.   A mixture of the γ_1- and γ_2-
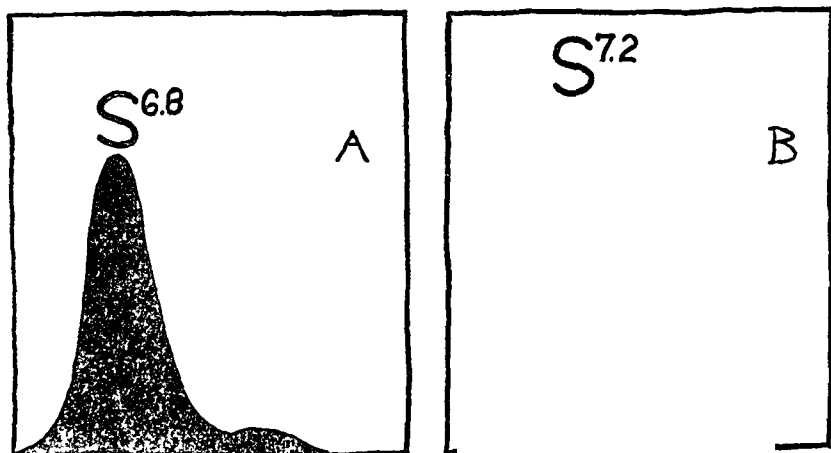
FIG. 6. Sedimentation patterns after 72 minutes at 220,000 times gravity of (A) γ_2-globulin fraction, (B) γ_1-globulin fraction.

globulins exhibited the molecular mass behavior to be expected of a system of this kind.

DISCUSSION

The proteins of horse serum which are concerned with antibody activity can be readily separated from the serum by means of ethanol fractionation. In this respect the γ-globulins from hyperimmune sera are more easily separated free of β-globulin than are those from normal sera.   The antibody fractions are relatively heterogeneous electrophoretically and may be separated into a series of fractions, the major components of which may show electrophoretic mobilities anywhere from -1.0 to -3.5×10^{-5} sq. cm. per volt per second in veronal buffer at pH 8.6 and ionic strength 0.1. Since these protein fractions show antibody activity, we have called them all γ-globulins in preference to using a series of unrelated terms such as γ, β_2, and T component (8), or γ, β_1, and β_2 (3), as has been done previously. The findings of Kekwick and Record are in agreement with the results of our work insofar as they may be compared.

The antibodies to the antigens we have studied appear to be distributed electrophoretically throughout this γ-globulin region. The γ_1- and γ_2-globulin fractions both contain antibody to a given antigen. The marked concentration of antibodies which make up only a small per cent of the total protein is not as readily achievable as it would be if the antibody were contained in a small and electrophoretically distinct component. The antibodies in question possess the same solubilities as do the γ_1- and γ_2-globulin serum components and, in order quantitatively to remove serum antibodies, these proteins must be likewise separated. Hence in preparing antibody-rich precipitates from horse serum the investigator is limited by the amount of antibody per unit of starting serum. Some slight difference in the solubilities of a given antibody is indicated by the results of the sub-fractionations of Precipitate A-B, as shown in Table II.

Fractionation of such protein systems must take into account isoelectric point and solubility distributions. For the antitoxins it would appear that, in agreement with previous findings, these antibodies are water-soluble and that furthermore they seem to be associated largely with the γ_1-globulins. Thus the γ_2-globulin fraction (Precipitate A-3B, Fig. 4) is much lower in antitoxin content than the water-soluble portion of the γ_1-globulin (see Table III). Thus Precipitate A-1B of Table II contains far more γ_1-globulin than does Precipitate A-2B but the antidiphtheritic potency of the latter fraction is far greater. This is apparently due to the γ_1-globulin of Precipitate A-2B being largely pseudoglobulin in nature as contrasted to the euglobulin characteristics of the globulin in Precipitate A-1B. As a further consequence of these fractionation conditions, the γ_2-globulins of Precipitate A-3B would tend to be more pseudoglobulin in nature than the analogous component in Precipitate A-2B. Such evidence is further indication that the antitoxic globulins of horse serum are more highly concentrated in the γ-globulins of lower isoelectric points and, as known before (20, 21), in the pseudoglobulin portions.

The bacterial antibodies as exemplified by the *pertussis* agglutinins show quite another behavior. Van der Scheer *et al.* (5) have indicated that bacterial antibodies appear to follow the γ_2-globulin component rather than the γ_1-component. The findings of Tiselius and Kabat (1), however, have indicated that horse pneumococcus antibody was a constituent corresponding to the γ_1-globulin (T component). Unfortunately, the two horses immunized in our work did not produce antibodies to the pneumococcus vaccine employed. It is difficult to reconcile the high content of *pertussis* agglutinin in both Precipitates A-A and A-3B (Table II). The former fraction is quite low in γ_1-globulins and very low in γ_2-globulins, while the Precipitate A-3B fraction is essentially all γ_2-globulin. The small amount of γ_1- and γ_2-globulin found by electrophoretic analysis in Precipitate A-A

and the low content of antitoxin are consistent. The euglobulin nature of Precipitate A-A is in agreement with the presence of the anticarbohydrate and certain antiprotein globulins of horse serum in the water-insoluble fractions (22–24). However, Precipitate A-3B is essentially water-soluble and likewise shows a large amount of *pertussis* agglutinin.

A further factor that must be considered is the degree of immunization that a particular animal has undergone. It must be realized, too, that both the chemical nature of the antigen and its route of administration are important factors in determining the characteristics of the antibody formed (24). The data of Fig. 3 clearly indicate a great deal of variation in the fractions obtained under analogous conditions from a single horse serum as the period of immunization progresses. Since the antibody fractions of the horse appear to be so complex, it would appear that a great deal of information would be gained by a careful and extended study of the physiology and rate of production of the serum proteins.

The horse γ-globulins separated in this work have sedimentation constants (s_{20w}) in the neighborhood of 7 Svedberg units. This figure is in agreement with previous data (2, 3, 25, 26) for horse globulin. No component having $s_{20w} = 18$ Svedberg units was observed to be present in our fractions. Small amounts of such heavy protein material have been found in the sera of apparently normal horses (25). The horse pneumococcus antibody (27, 28) is known to possess a sedimentation constant $s_{20w} = 18$ Svedberg units. The γ_2-globulin fractions gave values which were consistently slightly less than $s_{20w} = 7$ Svedberg units, while the γ_1-globulin fractions always gave a somewhat higher figure. Approximately 20 per cent of the protein sedimented at a rate corresponding to $s_{20w} = 8$ to 15 Svedberg units as a relatively polydisperse mixture. The γ_2-globulins from other animal plasmas also have been found to separate with varying amounts of this faster sedimenting material (15, 29, 30). It is not known whether such protein results from the fractionation conditions used or whether the γ-globulins exist as such in nature.

While the so called γ_1- and γ_2-globulin fractions were definitely heterogeneous on electrophoresis at pH 8.6, the peaks showed no tendency to give more than one main component over a wide pH range. As separated, the γ_1-globulin fractions were far more heterogeneous in nature than the γ_2-globulin fractions. However, the relatively homogeneous γ_2-globulin fraction obtained represents only a small portion of the serum proteins usually designated as γ_2-globulin (Fig. 1).

The ability of the β-globulins to separate more readily from hyperimmune sera is probably related to increased concentration of the γ-globulins. Thus while relatively constant amounts of β-globulin are precipitated in all sera, they represent a smaller per cent of the total globulin precipitated

from the immune systems. An evaluation of the amount of β-globulin in the serum or in an antibody fraction is difficult for, as seen from Fig. 1, it does not resolve well from the area described in an electrophoretic diagram as being due to γ_1-globulin. The very low content of antibody in such fractions as Precipitate A-A (Fig. 1) strongly suggests that antibody activity is not associated with β-globulin.

SUMMARY

An antibody-rich protein fraction of hyperimmunized horse plasma which is made up largely of γ_2- and γ_1-globulins may be readily separated from serum by ethanol fractionation. The antibodies appear to be distributed among molecules showing a wide variation in electrophoretic mobility. The largest amounts of antitoxin appear to be associated with the water-soluble portions of the γ_1-globulin fractions which is in contrast to the *pertussis* agglutinin.

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THE FATTY ACIDS OF CHLORELLA

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It has been found that the chemical composition of *Chlorella pyrenoidosa* varies over a wide range, depending on the environmental conditions selected for its growth. A description of the influence of different environmental factors on the composition of *Chlorella* has been given, and a method of calculating the approximate cell composition in terms of carbohydrate, protein, and lipide from the elementary analysis was developed (1). The most striking variation occurred in the calculated lipide content of the cells, which ranged from 5 to 85 per cent of the dry weight.

Little, if any, information is available on the composition of the fats of algae. The purpose of the work to be described here was to determine by analysis the lipide content of *Chlorella* grown under various conditions, to determine the composition of the lipide fraction, and, more particularly, to examine the fatty acids of *Chlorella*.

EXPERIMENTAL

Plant Material

The production of *Chlorella* having either extremely low or extremely high lipide content requires a much longer time and more specialized experimental conditions than those required to produce cells having between 20 and 75 per cent lipide. In order to obtain sufficient material for analysis of the lipide fraction of *Chlorella* cells having different lipide contents, large scale culture was undertaken to produce cells having about 20, 35, 60, and 75 per cent lipide.

Lot 1—A composite lot of cells was obtained from cultures grown in 5 gallon bottles in a greenhouse, as described elsewhere (2). The mineral nutrient medium contained 0.020 M $MgSO_4$, 0.018 M KH_2PO_4, 0.025 M KNO_3, and 0.000005 M $FeSO_4$ dissolved in previously boiled, cooled, and filtered tap water. The cultures grew for 80 days under natural illumination and were aerated with 5 per cent CO_2 in air. 1 kilo, dry weight, the yield from about 30 cultures, was used for lipide analysis.

Lot 2—Seven cultures, 15 liters each, in 5 gallon bottles were grown outdoors, near a north wall, for 17 days. The medium contained 0.010 M $MgSO_4$, 0.010 M KH_2PO_4, 0.000825 M $NH_4H_2PO_4$, 0.000715 M $(NH_4)_2HPO_4$, 0.030 M KCl, and 0.000005 M $FeCl_3$ dissolved in water pretreated as for Lot

1. 5 per cent CO_2 in air was bubbled through the cultures. The yield from seven cultures was 66.5 gm. dry weight.

Lot 3—The medium and conditions of growth were the same as for Lot 2, except that the time of growth was 83 days. The yield was 113 gm. dry weight from six 15 liter cultures.

Lot 4—Conditions known to produce cells of very high lipide content were employed in growing this lot of *Chlorella*. The medium was the same as in Lots 2 and 3. The gas stream was 5 per cent CO_2 in nitrogen. Ten cultures, each 2 liters in volume, were grown in Fernbach flasks. Each culture was illuminated continuously for 75 days by a 200 watt Mazda lamp 15 cm. below the flask. A water bath kept the temperature of the culture at 21–23°. The ten cultures yielded 51.6 gm. dry weight.

TABLE I

Composition of Chlorella

Lot No.	Found by analysis				Calculated		
	C	H	N	Ash	Carbo-hydrate	Protein	Lipide
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	48.64	6.88	6.80	7.71	33.4	46.4	20.2
2	53.46	7.66	4.15	4.97	37.5	27.3	35.2
3	60.95	8.80	2.01	4.69	23.5	13.1	63.4
4	65.58	9.43	1.23	3.49	15.0	7.9	77.1

Each culture was examined microscopically before the *Chlorella* cells were collected. No organism other than *Chlorella* was present in any of the cultures used in this work.

In order to show the difference in elementary composition of *Chlorella* of various lipide contents, and in order to check the validity of calculating the approximate carbohydrate, protein, and lipide content from the elementary analysis (1), these data for the four lots of *Chlorella* described above are shown in Table I.

Analytical Methods

Lipides—The extraction of lipides from dry *Chlorella* by anhydrous fat solvents is slow and usually incomplete. The extraction becomes quantitative and reasonably rapid if the dry plant material is first treated with water and then dehydrated with methanol. In this work, the dry, pulverized *Chlorella* was thoroughly mixed with twice its weight of water; then methanol was added slowly with continuous stirring until the alcohol concentration was 95 per cent by volume. The mixture was boiled 1 hour under a reflux, then filtered. The material was next extracted three times under a reflux with anhydrous methanol. Extraction was continued, methanol

and petroleum ether, b.p. below 70°, being used alternately until the material and extracts became colorless. A final extraction was made with ether. Usually no more than a trace of lipide was found in this last extract. The combined crude lipides from all the extractions were freed of solvent *in vacuo*. In order to remove the non-lipide materials which had been extracted by methanol, the crude lipide was taken up in anhydrous, alcohol-free ether and the solution filtered. Some of the non-lipide ether-insoluble material which was separated in this step was crystalline. The crystals were identified as sucrose. The ether was removed from the filtered lipide solution at reduced pressure and the lipide was dried to constant weight *in vacuo*. This material is reported as total lipide.

Because of the easily oxidizable nature of the extracted material, extracts were exposed to air as little as possible and were kept *in vacuo* between operations. All solvents were freshly redistilled just before use.

The total lipide from each lot of *Chlorella* was saponified by boiling 3 hours under a reflux with 5 per cent KOH in methanol, 10 ml. per gm. of lipide being used. Most of the methanol was then removed by distillation. The solution was diluted with water to its original volume. Unsaponifiable material was removed by thorough extraction with ether and its weight was determined after freeing it of solvent. The fatty acids were liberated by acidification of the aqueous solution with H_2SO_4 and were transferred quantitatively into low boiling petroleum ether. The solvent was removed at reduced pressure and the fatty acids were dried *in vacuo*.

Fatty Acids—The fractionation of the fatty acids was accomplished by the usual methods of lead salt separation and by distillation of the methyl esters. The total fatty acids from Lots 1 and 3 were first converted into methyl esters. The esters were fractionally distilled through a 12 inch Widmer column at 1 mm. pressure. Only a trace of ester distilled at a lower temperature than the boiling point of esters of the C_{16} acids. A sharp separation was obtained between esters of C_{16} and C_{18} acids. After distillation of the esters of C_{18} acids was complete, a residue amounting to 5 per cent of Lot 1 and 2 per cent of Lot 3 remained undistilled. Efforts to fractionate these residues were unsuccessful. They may have contained a small amount of esters of C_{20} or higher acids, plus decomposition products. The distilled esters were reconverted into the free acids, which were separated by the lead salt method into saturated and unsaturated fractions.

Lots 2 and 4 of *Chlorella* yielded an insufficient quantity of fatty acids to permit accurate separation of the methyl esters in the apparatus used for the larger lots. The acids from Lots 2 and 4 were not esterified, but were separated directly into saturated and unsaturated fractions by the lead salt method.

The equivalent weight and iodine number (Hanus) of each lot of total

fatty acids, and of each of the fractions of these, were determined. From these values the composition of the fatty acid mixture from each lot of *Chlorella* was calculated. The percentage of acids other than those of the C_{16} and C_{18} series was too small to be determined in the amount of material available and by the methods used. For that reason, the sum of C_{16} and C_{18} fatty acids in *Chlorella* is here taken to be 100 per cent.

TABLE II

Analysis of Lipide Fraction of Chlorella

Line No.	Analysis	*Chlorella* lot No.			
		1	2	3	4
1	Total lipide, % *of Chlorella*	23.37	33.17	62.96	75.51
	Composition of total lipide				
2	Fatty acids, % *of lipide*	28.0	49.5	83.0	86.8
3	Unsaponifiable, % *of lipide*	12.0	7.7	3.3	3.3
4	Water-soluble saponification products, % *of lipide*	60.0	42.8	13.7	9.9
5	Calculated fat, % *of Chlorella*	6.85	17.2	54.7	68.6
	Analysis of total fatty acids				
6	Iodine No. (Hanus)	163.1	143.8	143.6	125.3
7	Equivalent weight	269.5	273.6	272.7	274.1
8	Palmitic acid, % *of total*	16.6	10.9	7.9	11.4
9	Stearic acid, % " "	0.4	4.1	3.9	3.5
10	C_{16} unsaturated, % *of total*	29.1	18.3	27.3	18.0
11	C_{18} " % " "	53.9	66.7	60.9	67.1
	Degree of unsaturation				
12	C_{16} unsaturated acids	−4.1 H		−4.4 H	
13	C_{18} " "	−4.5 "		−3.4 "	
14	C_{16} + C_{18} unsaturated acids		−3.6 H		−3.2 H

RESULTS AND DISCUSSION

The analytical data for the lipides from the four lots of *Chlorella* are summarized in Table II. Comparison of the calculated lipide content shown in Table I with that found by analysis, Line 1 in Table II, shows good agreement, considering that the calculations assume a fixed composition of the lipide fraction, whereas analysis demonstrates that it varies between one lot of *Chlorella* and another.

Analysis of the lipides, Lines 2, 3, and 4 of Table II, shows a marked increase in the fatty acid content as the total amount of lipide increases, accompanied by a corresponding decrease in the unsaponifiable fraction. The increase in lipide content of *Chlorella* is mainly due to the accumula-

tion of fatty acids. No significant accumulation of hydrocarbons can have occurred.

It would have been of interest to determine the nature of the considerable quantity of water-soluble saponification products, Line 4, which were discarded. Chlorophyll degradation products could account for about half of this fraction in Lots 1 and 2. The chlorophyll content of *Chlorella* decreases rapidly as the lipide content increases. Lot 1 of *Chlorella* had 6 per cent dry weight of chlorophyll, while Lot 4 had only 0.03 per cent. Glycerol would constitute part of the material included in Line 4, a substantial part in Lots 3 and 4.

For comparison with the total lipide contents, Line 1, the fat contents were computed. The total fatty acids of each lot were calculated to triglycerides and the latter expressed as percentages of the *Chlorella*, Line 5.

As the lipide content of *Chlorella* increases, there is a significant decrease in the degree of unsaturation of the fatty acids, Line 6. The average molecular weight of the fatty acids, Line 7, is almost equal in Lots 2, 3, and 4, and is slightly lower in Lot 1. With the exception of the acids from Lot 1, the percentage of the different fractions, Lines 8 to 11, does not show a clearly defined relation to the change in fatty acid content of the *Chlorella*. Most of the saturated fatty acid is palmitic, with only a small amount of stearic acid.

The remarkable feature of the fatty acids from *Chlorella* is the great unsaturation of the liquid acids, Lines 12 to 14, particularly of the C_{16} fraction. The iodine number, 217.2, of the C_{16} unsaturated acids from Lot 3 requires the presence of at least 17 per cent of triply unsaturated acids in this fraction. Triply unsaturated C_{18} acid is required only in Lot 1 in order to account for the iodine number of that fraction. Comparison of the acids from Lots 1 and 3 shows that the over-all decrease in unsaturation can be attributed to the C_{18} acids, since the C_{16} acids of Lot 3 are even more unsaturated than those of Lot 1.

SUMMARY

Through control of environmental factors, four lots of *Chlorella pyrenoidosa* were grown, which contained 23, 33, 63, and 76 per cent lipide. The fatty acid content varied from 6 to 66 per cent of the dry weight of the cells. Analysis of the fatty acid mixtures showed that saturated acids, mostly palmitic, comprise 12 to 16 per cent of the total, and that the liquid acids are highly unsaturated.

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METHIONINE IN SELENIUM POISONING*

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The problem of selenium poisoning in farm animals has excited interest both from an academic and practical point of view. However, since the pioneering work of K. W. Franke and his coworkers, and the discovery of selenium in toxic grains by Robinson,[1] relatively less attention has been paid to the mode of action of selenium compounds or means of detoxification.

At an early date Hofmeister (1) reported that selenium salts were eliminated from the lungs in the form of volatile methylated derivatives. Inasmuch as the compounds were not isolated, this conclusion has been questioned (2, 3). The possibility that selenium may take the place of sulfur in the synthesis of mercapturic acids has been suggested by the observations of Moxon *et al.* (4). The feeding of bromobenzene to selenized steers resulted in a reduction of the selenium content of the blood with a corresponding rise in urinary selenium. Other workers (5), however, were not able to duplicate these findings in rabbits. The observation that arsenite is capable of alleviating selenium toxicity in animals is at present inexplicable (6).

The present communication deals primarily with the toxicity of selenate and its counteraction by methionine in yeast.

EXPERIMENTAL

The organism used was *Saccharomyces cerevisiae* (Fleischmann) which was transferred daily on molasses agar.[2] The medium used throughout the study was a modification of that used by Williams and Saunders (7) with

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[1] For an excellent review on selenium poisoning the reader is referred to Painter's monograph (Painter, E. P., *Chem. Rev.*, 28, 178 (1941)).

[2] The molasses agar has the following composition: 4 per cent molasses, 0.12 per cent $NH_4H_2PO_4$ and 2 per cent agar.

the same organism. Since preliminary experiments indicated that the extent of inhibition depended upon the amount of sulfate present (cf. analogy in plants (8, 9)), the sulfate content was reduced to 0.5 gm. of $Na_2SO_4 \cdot 10H_2O$ per liter of medium. This concentration permitted optimum growth of yeast in the absence of selenate, but was sufficiently low to exert no influence on the inhibition caused by 1 mg. of selenate per tube. The double strength medium is given in Table I, where the percentages in parentheses are those of sulfur present as contamination.

All tests were carried out in 20×150 mm. lipless Pyrex test-tubes, which received 5 ml. of the double strength medium, followed by any ingredients to be tested, and finally with water to a volume of 10 ml.

TABLE I

Basal Medium for Saccharomyces cerevisiae (Fleischmann)

Sucrose	40	gm.	(0.000 %)	FeCl₃	1.0 mg.
(NH₄)₂ tartrate	6	"	(0.001 %)	CuCl₂	0.2 "
KH₂PO₄	4	"	(0.000 %)	KI	0.2 "
Asparagine	3	"	(0.000 %)	Inositol	5 "
Na₂SO₄·10H₂O	0.5	"		Thiamine hydrochloride	40 γ
CaCl₂·2H₂O	0.5	"	(0.01 %)	β Alanine or calcium panto-	
MgCl₂	0.5	"	(0.000 %)	thenate	0.5 mg.
TlCl₃	2	mg.		Pyridoxine	40 γ
ZnCl₂	2	"		Biotin	1 "
MnCl₂	2	"		Distilled H₂O to	1000 ml.
H₃BO₃	2	"		pH	5.3

The tubes were sterilized by steaming for 15 minutes, and inoculated when cool. It was found that the maintenance of sterility could be accomplished by covering the tubes with a sterile towel instead of the usual cotton plugs.

For an inoculum, four to five loopfuls of yeast from a freshly grown slant were homogeneously suspended in 15 ml. of sterile saline. This was centrifuged and resuspended in another 15 ml. of saline. The yeast concentration as determined turbidimetrically was approximately 0.8 mg. per ml. on a wet weight basis. 1 drop of this suspension was used as the inoculum, although it was found that the size of the inoculum was not critical; 5 drops yielded the same results as 1. The tubes were incubated at 30–31° for 2 to 3 days and the turbidity was measured in a photoelectric turbidimeter with a 5400 A filter. Growth was expressed in terms of optical density which is equal to log 100 minus per cent transmission $(2 - \log G)$.

Reversal of Selenate Toxicity by Methionine

Preliminary experiments showed that hydrolyzed casein was capable of reversing the inhibition produced by 1 mg. of selenate[3] per culture of yeast. By using the individual amino acids in 2 mg. quantities per tube, it was found that only methionine (the DL variety was employed) possessed such activity. Negative results were obtained with the following substances: L-tryptophan, DL-isoleucine, L-leucine,[4] DL-alanine, DL-phenylalanine, L-histidine, DL-valine, glycine, DL-glutamic acid, DL-serine, L-arginine, L-cystine, or 2 mg. of a mixture of these amino acids. Negative results were also obtained with 2 mg. of each of the following: L-cysteine,[5] gluta-thione,[5] DL-ethionine,[6] thymine, cytosine, guanosine, guanylic acid, a-denosine, adenylic acid, 2-methyl-6-aminopyrimidine, 2-methyl-5-ethoxy-methyl-6-aminopyrimidine, hydrolyzed desoxyribonucleic acid[7] as prepared by Levene and Bass (14), and 200 γ of each of the following: 2-amino-4-hydroxy-6-methylpyridine, 2-amino-4-hydroxypyridine-6-carboxylic acid, and pteroic acid.

The relationship of DL-methionine to selenate inhibition is depicted in Table II and shown graphically in Fig. 1. Straight lines are obtained when the plot is made on a logarithmic scale. Concentrations of methionine above 3 mg. per tube did not enhance the reversal.

In view of the fact that animals can utilize homocystine plus a methyl donor such as choline or betaine (15, 16) in lieu of methionine, an attempt was made to determine whether yeast can use these substances to reverse the effect of selenate. No activity was noted with 2 mg. of DL-homocystine, choline, betaine, or creatinine, either individually or in combination.

Action of Methionine-Free Casein Hydrolysate

The specificity of methionine reversal was also tested by noting the effect of a methionine-free casein hydrolysate upon selenate activity. The

[3] The authors are indebted to E. P. Eddy and W. E. Caldwell for the gift of 87.2 per cent H_2SeO_4 prepared by the method of Gilbertson and King (10). In all these experiments the pH of all ingredients was adjusted to that of the medium before use.

[4] Partial activity was obtained with one sample of leucine (Eastman). However, a sodium fusion yielded a positive sulfur test, and the sample gave a positive test for methionine by the McCarthy-Sullivan method (11). Previous authors (12, 13) have warned of methionine contamination of leucine in isolation products. When synthetic leucine was used (Merck), no reversal was noted.

[5] L-Cysteine and glutathione reacted with H_2SeO_4 to form elemental Se. This took place even prior to the inoculation, but no reversal was evidenced.

[6] Kindly furnished by United States Industrial Chemicals, Inc.

[7] Obtained from the Krishell Laboratories, Portland, Oregon.

hydrolysate was prepared as follows, with a modification of Baernstein'
method for determining methionine in protein hydrolysates (17): 500 mg
of Casamino acids (Difco) were refluxed with 25 ml. of freshly distilled H
(sp. gr. 1.7) for 8 hours in a CO_2 atmosphere. The excess HI was remove
by vacuum distillation, a few drops of dilute HCl being used to facilitat
removal. A silver chloride suspension (prepared by precipitating Ag₂(

TABLE II

Relationship of DL-*Methionine to Selenate Inhibition in Saccharomyces cerevisi*
Incubation time, 52 hours.

H_2SeO_4	Methionine	Optical density
mg.	*mg.*	
0	0	0.980
1	0.0	0.000
1	0.5	0.381
1	1.0	0.718
1	2.0	0.770
1	3.0	0.842
2	0	0.000
2	0.5	0.232
2	1.0	0.660
2	2.0	0.732
2	3.0	0.785
3	0	0.000
3	0.5	0.212
3	1.0	0.640
3	2.0	0.650
3	4.0	0.708
4	0	0.000
4	0.5	0.164
4	1.0	0.631
4	2.0	0.680
4	3.0	0.702
5	0	0.000
5	0.5	0.125
5	2.0	0.675

from $AgNO_3$ and NaOH, centrifuging, decanting the supernatant, and
adding strong HCl to form AgCl) was added in excess to the hydrolysate.
This was added to a large centrifuge tube, shaken for 10 minutes, centri-
fuged, and filtered. The precipitate was washed twice and the washings
were added to the filtrate. The latter was evaporated to dryness and
reconstituted to 50 ml. The final concentration was thus 10 mg. per ml.
The filtrate gave a negative McCarthy-Sullivan test for methionine.

The effects of this hydrolysate, with and without supplementation with DL-methionine, are summarized in Table III. No reversal of selenate inhibition was obtained with this preparation, but when DL-methionine
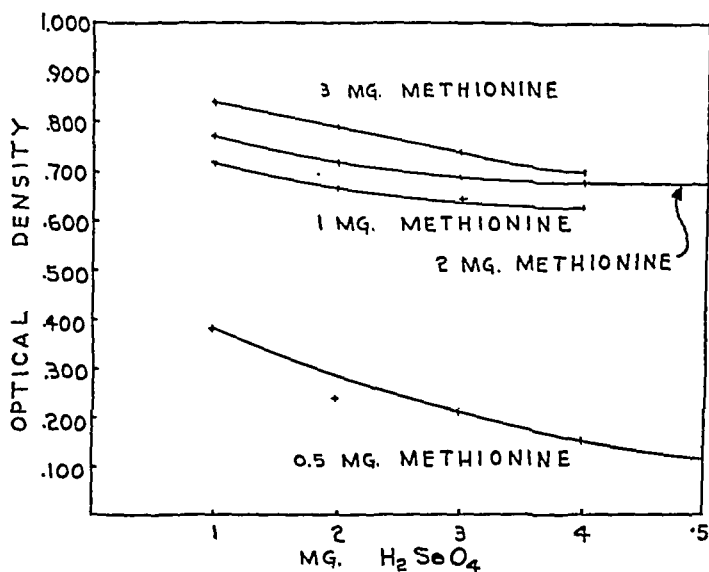
FIG. 1. Reversal of selenate inhibition by DL-methionine

TABLE III

Effect of Methionine-Free Casein Hydrolysate upon Selenate Inhibition in Yeast
Incubation time, 71 hours.

H₂SeO₄	DL-Methionine	Methionine-free hydrolysate	Casein hydrolysate	Optical density
mg.	*mg.*	*mg.*	*mg.*	
0	0	0	0	1.32
1	0	0	0	0.000
1	2	0	0	1.03
0	0	10	0	1.27
1	0	10	0	0.00
1	2	10	0	1.16
0	0	0	28.5	1.48
1	0	0	28.5	0.41
1	2	0	28.5	1.34

was also added the growth of yeast seemed fully restored. Although the levels employed do not permit a precise calculation of the reversing power of ordinary casein hydrolysate in terms of methionine content, it is evident that the casein effect is due principally, if not entirely, to methionine.

In Vitro Oxidation of Methionine by Selenic Acid

The possibility exists that the mode of action of selenate as a toxic reagent for organisms rests on the oxidative capacity of the selenate. Methionine is known to be oxidized readily to the sulfoxide (18) or the sulfone (19) with various oxidizing agents. These have been shown to repress the growth of *Lactobacillus arabinosus* and *Lactobacillus casei* (20, 21). Similarly, the rat has been shown incapable of substituting the sulfone for methionine in the diet (22). If this is true for selenate then its action could

TABLE IV

Specificity of L-Methionine in Selenate Inhibition

Incubation time, 67 hours.

H_2SeO_4	DL-Methionine	Optical density	L-Methionine	Optical density	D-Methionine	Optical density
mg.	mg.		mg.		mg.	
0	0	1.120				
1	0	0.000	0.25	0.558	0.25	0.000
1	0.25	0.494	0.50	0.881	0.50	0.000
1	0.50	0.828	1.00	0.910	1.00	0.000
1	1.00	0.839	2.00	0.981	2.00	0.000
1	2.00	0.980				
2	0.25	0.187	0.25	0.257	0.25	0.000
2	0.50	0.770	0.50	0.980	0.50	0.000
2	1.00	0.805	1.00	0.850	1.00	0.000
2	2.00	0.860	2.00	0.920	2.00	0.000
3	0.25	0.000	0.25	0.045	0.25	0.000
3	0.50	0.070	0.50	0.144	0.50	0.000
3	1.00	0.655	1.00	0.720	1.00	0.000
3	2.00	0.710	2.00·	0.760	2.00	0.000

be explained by its prevention of methionine utilization. An attempt was therefore made to oxidize methionine with selenic acid. It was found that methionine underwent a ready reaction with selenic acid at room temperature with the rate depending upon the concentrations of starting materials. A crystalline substance containing Se, N, and S was isolated and a description of the procedure will be dealt with in a future paper.

Specificity of L-Methionine in Reversal of Selenate

The postulate has recently been made (23) that there is present in mammalian tissue a heat-labile factor or factors, presumably an enzyme, which is responsible for the decomposition of selenate and selenite. This is based on the observation that fresh tissue can decompose the selenium salts, whereas autoclaved tissue is incapable of such action. We have ob-

tained somewhat similar results with yeast. This action lends to the cell an active rôle in the detoxification mechanism, in which methionine might possibly cooperate. In order to gain evidence on this point, DL-methionine was resolved into its optical isomers and each was tested for its ability to reverse the selenate effect.

The resolution was carried out according to the method of Windus and Marvel (24). The D-methionine obtained had a specific rotation[8] $[\alpha]_D^{25}$ = +6.88° (0.2000 gm. in 25 ml. of H_2O, 2 dm. tube). The L-methionine obtained had a specific rotation[8] $[\alpha]_D^{25}$ = −5.0 (0.2008 gm. in 25 ml. of H_2O, 2 dm. tube). Both isomers gave a positive McCarthy-Sullivan test, but *Lactobacillus arabinosus* utilized only the L form (26) with Henderson and Snell's medium for methionine determination (27).

The results listed in Table IV show clearly that only the natural isomer of methionine is capable of reversing the inhibition by selenate, although the racemic mixture is somewhat more than 50 per cent as active as equal amounts of the L form. This might be due to incomplete resolution (*cf.* foot-note 8); but if not, it suggests that the natural isomer promotes the utilization of the unnatural one, an observation which has also been made for glutamic acid in *Lactobacillus arabinosus* (28).

DISCUSSION

The specificity of methionine in the detoxification of selenate by yeast is apparent first from its direct relationship as depicted in Fig. 1. Within limits, the growth of yeast in selenate is directly proportioned to the amount of methionine present. That methionine is the only substance capable of such action is seen both from the numerous substances tested and the experiments with methionine-free casein hydrolysate. It is interesting to note that unlike animal tissues, yeast is incapable of utilizing homocystine and a methyl donor such as choline, betaine, or creatinine. This would indicate that methionine is synthesized by yeast in a different manner from that accomplished by animals. The fact that yeast is able to synthesize its sulfur-containing amino acids from the sulfate provided lends support to this concept.

The results obtained with the optical isomers of methionine indicate that the cell plays an active rôle in the detoxification mechanisms. This is in line with the recent work of Rosenfeld and Beath (23) who postulated the existence of a selenate-decomposing enzyme in mammalian tissue. Although it is not known whether methionine is involved in the latter system, this compound appears to be the agent by which selenate is detoxified in

[8] D-Methionine $[\alpha]_D^{25}$ = +7.1° obtained by Jackson and Block (25), +8.12° (Windus and Marvel (24)); L-methionine $[\alpha]_D^{25}$ = −7.3° (Jackson and Block (25)), −6.9° (from casein, Windus and Marvel (24)), −7.5° (synthetic product, Windus and Marvel (24)).

yeast, and the specificity of the reversal process is of a type usually associated with enzymatic activity.

The mechanism of the toxic effect of selenium salts remains to be solved. It is believed that the selenate must previously be reduced to selenite before inhibition can take place (29, 23). The effect of the selenite upon vital cellular components has not been explained satisfactorily. The succinic dehydrogenase system has been found to be completely inhibited by selenite (29, 30). Potter and Elvehjem (31) have found that O_2 uptake by yeast in glucose, fructose, or mannose but not in lactose or pyruvate is inhibited by selenite. These authors believe that the selenium acts primarily as an inhibitor of the glycolytic system, possibly by restricting the utilization of glutathione. Since the function of the latter is not known, this explanation is of limited value. DuBois, Rhian, and Moxon (32) have been able to reverse the toxic effect of selenate in rats with glutathione. We have not observed this effect with yeast. Finally, it would seem that the succinic dehydrogenase system, if present in yeast, plays a relatively unimportant rôle, since these organisms utilize succinic acid only poorly.

In view of the ready ability of selenic acid to react with methionine *in vitro*, the transformation of the latter into a compound non-utilizable by the cell offers an attractive explanation for the toxic effect of selenate. However, when dilute concentrations of the reactants are used, the reaction is slow at room temperature. This could not therefore explain the immediate inhibition of O_2 uptake in yeast as observed by Potter and Elvehjem (31).

From the observation that methionine never produces complete reversal of the inhibition, it can be inferred that probably more than one system is affected by the selenate.

From a practical point of view, it is of interest to note that methionine affords protection against selenite in rats (33). It is believed that, in the light of the present study, methionine should be investigated in combatting selenium poisoning in farm animals.

SUMMARY

1. Inhibition of yeast growth by selenate has been found to be reversed by the addition of methionine. No other compound tested displayed this reversal.

2. Within limits, the growth of yeast in the presence of selenate is directly proportional to the methionine present.

3. A methionine-free casein hydrolysate was prepared, and found to have no reversing effect. The addition of DL-methionine rendered it active.

4. Normal casein hydrolysate was capable of partially reversing the selenate inhibition. The addition of methionine enhanced this activity.

5. Yeast could not utilize DL-homocystine plus a methyl donor such as choline, betaine, or creatinine to reverse the inhibition. This suggests that methionine is synthesized in a manner different from that in mammalian tissue.

6. An *in vitro* reaction between selenic acid and methionine was found to take place readily at room temperature. A crystalline reaction product was obtained which contained Se, N, and S.

7. Of the optical isomers of methionine, only the naturally occurring L form was active. This indicates active participation of the cell in the detoxification mechanism.

8. A brief discussion is given of previously proposed explanations for the mode of action of selenium compounds.

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THE IN VITRO SYNTHESIS OF CHOLESTEROL FROM ACETATE BY SURVIVING ADRENAL CORTICAL TISSUE*

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The prominence of the liver as a site of cholesterol synthesis has been demonstrated by Bloch and his associates (1, 2). Recent observations in this laboratory have shown, however, that the conversion of acetic acid to cholesterol can occur in the hepatectomized rat.[1] In seeking to localize the extrahepatic tissue or tissues in which this synthesis occurs, attention was at first directed to the adrenal cortex because of its high cholesterol content (3). That this tissue is indeed a site of active cholesterol synthesis is borne out by the findings presented here.

EXPERIMENTAL

Preparation and Treatment of Adrenal Cortical Slices—Beef adrenal glands were used in the present investigation. The glands were excised at the abattoir 15 to 20 minutes after the animals had been killed, immediately wrapped in water-proof cellophane, and immersed in ice water for transport to the laboratory. The glands were first freed of extraneous tissue, split in half, and then carefully demedullated. The cortex was next sliced free-hand with a razor blade and the slices transferred to a dish containing cold (0°) bicarbonate-Ringer's solution prepared according to Krebs and Henseleit (4). 500 mg. of slices were gently blotted on moist filter paper, weighed quickly, and transferred to a 50 cc. glass-stoppered Erlenmeyer flask containing 5 cc. of the bicarbonate-Ringer's solution and 0.5 cc. of 0.024 N $C^{14}H_3C^{14}OONa$ having 9.4×10^5 counts per minute per cc.[2] The bicarbonate-Ringer's solution was saturated with a gas mixture consisting of 5 per cent CO_2 and 95 per cent O_2, and its pH adjusted to 7.4 to 7.5 just before the addition of the slices. The atmosphere in the flasks was saturated with the same gas mixture immediately before and $1\frac{1}{2}$ hours

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[1] Burstein, L. S., Srere, P. A., and Chaikoff, I. L., unpublished observations.

[2] We are indebted to Dr. H. A. Barker for the doubly labeled acetate used in this investigation. It was prepared from $C^{14}O_2$ with the aid of *Clostridium thermoaceticum* (5).

after the flasks were placed in a constant temperature water bath maintained at 37.5°.   The slices were incubated for 3 hours.

Isolation of Cholesterol—At the end of the incubation period the contents of the flasks were transferred to an alcoholic KOH solution (15 gm. of KOH in 50 cc. of 95 per cent ethyl alcohol for each 10 gm. of tissue) and the mixture refluxed for 8 hours on a steam bath.   The alcohol was evaporated at steam bath temperature and the alkaline hydrolysate exhaustively extracted with petroleum ether.   To insure efficient extraction of the lipides, it was found necessary to keep the ratio of petroleum ether to the alkaline hydrolysate 10:1.   Four extractions proved sufficient to remove all of the non-saponifiable fraction; the amount of digitonin-precipitable material extractable from the alkaline hydrolysate was negligible after it had been subjected to four extractions with petroleum ether.   The petroleum ether extracts were combined, dried over Na_2SO_4, filtered, and evaporated to dryness on a steam bath.   The residue was then dissolved in a minimum amount of hot ethyl alcohol, and an excess of 1 per cent digitonin (in 80 per cent ethyl alcohol) was added.   The mixture was heated for an additional 5 minutes and then allowed to stand overnight at 5°.   The digitonide was filtered and then washed successively with 85 per cent alcohol, 1:2 acetone-ether mixture, and finally with anhydrous ether.   The digitonide was dried and stored in a vacuum desiccator.   The C^{14} content of the digitonide was then determined.

The digitonide was cleaved by the pyridine method (6).   The crude cholesterol obtained was acetylated with acetic anhydride and the acetate recrystallized once from aqueous methanol.   The radioactive cholesteryl acetate obtained was weighed and mixed with a known amount of non-radioactive cholesteryl acetate (the dilution factor was 11.7).   The mixture was then recrystallized twice from aqueous methanol.   The C^{14} content of the cholesteryl acetate was then determined.

The cholesteryl acetate was saponified with methanolic sodium hydroxide and the cholesterol isolated by dilution with water.   The cholesterol isolated was weighed and its C^{14} content determined.

Isolation of Fatty Acids—The aqueous alkaline fraction that remained after the petroleum ether extractions was made acid to bromocresol green and the precipitate allowed to settle.   This precipitate has been shown by Sperry *et al.* (7) to contain all the fatty acids of the fraction.   In confirmation of Sperry's observation, it was found that the aqueous filtrate contained negligible amounts of ethyl ether-extractable material or of C^{14}.   The supernatant was decanted and the residue filtered.   The precipitate obtained was exhaustively extracted three times with acetone.   The acetone extracts were then combined and evaporated to dryness in the presence of a CO_2

atmosphere, and the residue obtained was extracted with hot petroleum ether. The petroleum ether-insoluble fraction was now redissolved in acetone, and the above procedure repeated. The two petroleum ether extracts were combined, dried over Na_2SO_4, and filtered. The fatty acid content in the petroleum ether extract was determined by weight and titration. The C^{14} content of these fatty acids was determined as described below.

Determination of C^{14}—Samples were oxidized by the method of Van Slyke and Folch (8). The $C^{14}O_2$ evolved was collected as $BaC^{14}O_3$ and its C^{14} content determined by the method of Dauben *et al.* (9).

TABLE I

Recovery of C^{14} in Fractions Isolated from Beef Adrenal Cortex after Incubation with $C^{14}H_2C^{14}OOH$

Experiment No.	Adrenal cortex tissue in bath	C^{14} acetate added to Ringer's solution	Fraction or compound isolated	Total counts recovered in compound or fraction	Specific activity	Per cent of added C^{14} recovered in compound or fraction
	gm.	counts				
1	21	1.9×10^7	Cholesterol digitonide	3.5×10^4	1840*	0.2
			Fatty acids	2.0×10^5	400	1.0
			Aqueous residue	8.4×10^5		4.4
2	11	9.4×10^6	Cholesterol digitonide	3.4×10^4	1830*	0.4
			Fatty acids	2.8×10^4	150	0.3
			Aqueous residue	5.3×10^5		5.6

* Refers to counts per minute per mg. of its cholesterol.

Results

After incubation, the adrenal cortex tissue and the contents of the flasks were separated into three fractions: cholesterol, fatty acids, and aqueous residue. The C^{14} recovered in these fractions is recorded in Table I. In the first experiment 0.2 per cent of the added C^{14} was converted to cholesterol and 1.0 per cent to fatty acids; in the second experiment 0.4 and 0.3 per cent, respectively, were so converted.

The melting points found for cholesterol and its acetate were in good agreement with reported values (Table II).

The values for the specific activities of cholesterol and of its two derivatives, the acetate and the digitonide, are recorded in Table II. The specific activity of the digitonide is expressed as counts per minute per mg. of the digitonide in Column 4 and as counts per minute per mg. of its cholesterol in Column 6.

Since non-radioactive cholesteryl acetate had been added to the radio-

active cholesteryl acetate isolated from the adrenal cortex, it was necessary to correct the values obtained for the specific activity of the acetate and of the cholesterol by the dilution factor 11.7 in order to compare their specific activities with that of the digitonide. The corrected values are recorded in Column 6 of Table II.

TABLE II

Showing Degree of Constancy in Specific Activity of Recrystallized Cholesterol and of its Derivatives

Compound isolated	Melting point		Specific activity		
	Determined	Literature	Expressed as counts per min. per mg. of compound isolated	Expressed as counts per min. per mg. of cholesterol in compound isolated	Expressed as counts per min. per mg. of isolated adrenal cortex cholesterol
(1)	(2)	(3)	(4)	(5)	(6)
Cholesterol digitonide	°C.	°C.	4.4×10^2	1.8×10^3	1.8×10^3
Cholesteryl acetate	113–114	115–116	1.4×10^2	1.6×10^2	1.9×10^3
Cholesterol	147–148.5	148–149	1.4×10^2	1.4×10^2	1.7×10^3

DISCUSSION

The constancy of the specific activities of cholesterol and of its two derivatives, the acetate and the digitonide (Column 6, Table II), whose purity had been established (Column 2, Table II), provides conclusive evidence that the C^{14} of the doubly labeled acetic acid had been incorporated into cholesterol of the adrenal cortex.

The demonstration that an isolated surviving tissue can convert acetate to cholesterol was first shown for the liver by Bloch and his associates [1]. He also reported that no such synthesis took place in slices of kidney, testis, spleen, and gastrointestinal tract [1]. The results of the present investigation demonstrate that another tissue, namely one concerned with the secretion of a steroidal hormone, is capable of converting the common metabolic intermediate acetate to cholesterol.

SUMMARY

The conversion of C^{14}-labeled acetate to cholesterol by surviving slices of beef adrenal cortex is demonstrated. The presence of C^{14} in the cholesterol molecule was established by the finding of a constant specific activity in the cholesterol and in two of its derivatives, the digitonide and the acetate.

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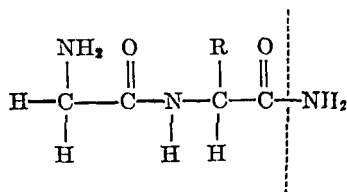
THE SPECIFICITY OF LEUCINE AMINOPEPTIDASE*

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Since Linderstrøm-Lang's demonstration that the hydrolysis of L-leucylglycine (LG) is due to a distinct leucyl peptidase (1), various studies have shown that this enzyme is widely distributed (2–4) and requires for its activity the presence of Mn^{++} or Mg^{++} ions (2, 5). The enzyme has been regarded as a typical aminopeptidase (5), since it does not hydrolyze acylated compounds such as benzoyl-L-leucylglycine, and since, in addition to the dipeptide, it hydrolyzes L-leucinamide (LA) and the tripeptides, L-leucylglycylglycine (LGG) and L-leucyl-L-leucylglycine.

It has now been observed that a highly purified preparation of leucine aminopeptidase from hog intestinal mucosa can hydrolyze glycyl-L-leucinamide (GLA). Under the conditions of our experiments, the reaction ceases after the decomposition of a single peptide bond. Since glycyl-L-leucine is not appreciably hydrolyzed, the hydrolysis must occur at the terminal amide bond, as indicated by the dotted line; R represents the

$$
\begin{array}{ccccc}
\mathrm{NH_2} & \mathrm{O} & & \mathrm{R} & \mathrm{O} \\
| & \| & & | & \| \\
\mathrm{H-C} & \mathrm{-C-N-C-C} & \vdots & \mathrm{-NH_2} \\
| & & | \quad | & & \\
\mathrm{H} & & \mathrm{H} \quad \mathrm{H} & &
\end{array}
$$

isobutyl side chain. Thus, the products of the reaction must be glycyl-L-leucine and ammonia. If the action occurred at the other peptide bond, liberating glycine and LA, the second peptide bond would also be split, since LA is rapidly hydrolyzed by the enzyme. The observation that GLA is hydrolyzed by leucine aminopeptidase has been possible only because the purification process has removed the glycyl-L-leucine dipeptidase. With crude extracts of hog intestinal mucosa and other tissues, the consecutive hydrolysis of both peptide bonds occurs (6).

Table I shows that the hydrolysis of GLA follows the kinetics of

<div align="center">

TABLE I

Specificity of Leucine Aminopeptidase

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The enzyme was incubated at 40° in veronal buffer at pH 7.8 to 8.0 with 0.01 M Mn++ for 3 hours prior to addition to the buffered substrate solution (0.05 M). The proteolytic coefficient $C = K/E$, where K is the first order velocity constant for the enzyme concentration E expressed in mg. of protein N per cc. of test solution. Two different enzyme preparations of somewhat different activities were used; these are distinguished by the letters in parentheses following the substrates.

Substrate	Enzyme concentration	Time	Hydrolysis	C	C, average
	γ protein N per cc.	hrs.	per cent		
L-Leucinamide (a)	0.8	0.5	19	3.7	3.9
		1.0	36	4.1	
		1.5	46	3.8	
		2.0	58	4.0	
		2.5	66	3.9	
Glycyl-L-leucinamide (a)	0.8	0.5	13	2.5	2.2
		1.0	18	1.8	
		1.5	30	2.2	
		2.0	39	2.2	
		2.5	46	2.2	
L-Leucylglycine (a)	0.8				5.5
L-Leucylglycylglycine (a)	0.8				6.5
Glycyl-L-leucine (a)	0.8	3.0	0		
"	4.0	3.0	2		
Diglycylglycine (a)	4.0	3.0	0		
L-Leucinamide (b)	1.31				2.3
Carbobenzoxy-L-leucinamide (b)	6.55	20	1		
Glycyl-L-leucinamide (b)	1.31				1.5
Carbobenzoxyglycyl-L-leucinamide (b)	6.55	20	1		
L-Glutamyl-L-leucinamide (b)	1.31				1.5
Carbobenzoxy-L-glutamyl-L-leucinamide (b)	6.55	20	3		
L-Leucyl-L-glutamic acid (b)	1.31				1.1
Glycylglycyl-DL-leucylglycine* (b)	6.55				0.13
Carbobenzoxyglycylglycyl-L-leucinamide (b)	6.55	20	1		
Carbobenzoxy-L-leucylglycylglycine (b)	6.55	20	0		
Carbobenzoxy-L-leucylglycine (b)	6.55	20	0		

* Used at 0.1 M. We are indebted to Dr. J. S. Fruton for a sample of this compound.

order reaction similar to those of the other substrates.[1] The hydrolysis of all of the sensitive compounds is strongly activated by Mn++, and the

[1] The hydrolysis of LGG follows the kinetics of a zero order reaction. The approximate initial first order constant is given for comparison with the other substrates.

activation of the enzyme by Mn^{++} follows the same type of time reaction for GLA as for the other substrates (5).

Because of our finding that leucine aminopeptidase hydrolyzes an amide linkage at a distance from the free amino group, as in GLA, the possibility that the enzyme may possess some endopeptidase activity was investigated. Fruton and Bergmann (7) have reported that chymotrypsin shows a dual specificity and can act on substrates characteristic both of endopeptidase and aminopeptidase specificities. We have, therefore, tested the action of our enzyme on a variety of substrates. These results are also presented in Table I.

It is clear that leucine aminopeptidase does not have any detectable endopeptidase action, since no hydrolysis could be observed with carbobenzoxyglycyl-L-leucinamide, carbobenzoxyglycylglycyl-L-leucinamide, carbobenzoxy-L-glutamyl-L-leucinamide, or other N-acylated peptides. On the other hand, compounds with a free amino group such as L-leucyl-L-glutamic acid and L-glutamyl-L-leucinamide (GlLA) are readily hydrolyzed. With GlLA only one peptide bond is hydrolyzed when the purified enzyme is used. However, with a crude extract of intestinal mucosa, both bonds are rapidly hydrolyzed.

It is of interest that the enzyme preparation has a slow but definite action on glycylglycyl-DL-leucylglycine (GGLG). One must assume that this action is due to the leucine aminopeptidase, since no action was detected on diglycylglycine.

DISCUSSION

It is now possible to define the specificity requirements of leucine aminopeptidase more precisely than heretofore. The data in Table I show that the residues on the carboxyl end of the leucine group have some influence on the rate of hydrolysis. The most rapid action is on the tripeptide (LGG) and the dipeptide (LG). The markedly slower action on L-leucyl-L-glutamic acid as compared with LA indicates some inhibitory effect of the second carboxyl group when it is near the sensitive bond. On the other hand, the general configuration of the moiety attached to the carboxyl end of the leucine residue cannot be highly critical, since it has been demonstrated that L-leucyl-β-alanine is rapidly hydrolyzed by this enzyme (8).

The finding of a rapid action on GLA and GlLA was somewhat unexpected. Nevertheless, in the presence of the free amino group, 1 residue removed from the sensitive peptide bond does reduce the rate of hydrolysis by about 40 per cent as compared with LA. The much slower action on the tetrapeptide, GGLG, indicates that the sensitivity of the substrate is greatly decreased as the distance between the sensitive peptide bond and the free amino group is increased.

Leucine aminopeptidase has hitherto been regarded as the prototype of an aminoexopeptidase (9). This concept must now be revised to include our finding that the free amino group need not be present on the leucine residue which possesses the sensitive peptide bond. It has been recently suggested (10) that the rôle of heavy metals in peptidase action is the formation of a coordination compound linking the enzyme and the substrate. If this is so, one must assume that the ease with which the metal (Mn^{++} or Mg^{++}) can form this bridge is a critical function of the distance from the sensitive bond.

EXPERIMENTAL

The enzyme experiments were performed as described in previous papers from this laboratory (4, 8) by means of the carboxyl titration method of Grassmann and Heyde (11). The leucine aminopeptidase was purified in the manner described by Smith and Bergmann (5). Some further purification was achieved by precipitation of the enzyme with 33 per cent acetone at room temperature, followed by dialysis and removal of the inactive precipitate. This procedure gave preparations essentially free of glycyl-L-leucine dipeptidase and tripeptidase activity as measured on diglycylglycine and prolidase.

L-*Leucinamide Hydrochloride*[2]—A recrystallized preparation of L-leucine methyl ester hydrochloride (10 gm.) was allowed to stand in a pressure bottle at room temperature for 2 days with 50 cc. of anhydrous methanol which had previously been saturated with ammonia gas at 0°. The solution was then repeatedly concentrated *in vacuo* with methanol, and the crystals were filtered and washed with ether. Yield, 8.5 gm. After recrystallization from methanol-ether, thin plates were obtained; m.p. 236–237°.

$$C_6H_{15}ON_2Cl. \quad \text{Calculated.} \quad C\ 43.3,\ H\ 9.1,\ N\ 16.8$$
$$166.6 \qquad \text{Found.} \qquad ``\ 43.3,\ ``\ 9.1,\ ``\ 16.9$$
$$[\alpha]_D^{25} = +9.5°\ (5\%\ \text{in water})$$

Behrens and Bergmann (12) found $[\alpha]_D^{27} = +9.25°$ for the corresponding acetate obtained by hydrogenation of carbobenzoxy-L-leucinamide.

Glycyl-L-*leucinamide Hydrochloride*

Carbobenzoxyglycyl-L-*leucine Methyl Ester*—This compound has previously been described as an oil (13). The coupling was performed in the

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[2] Although this compound has previously been described as the acetate obtained by hydrogenation of carbobenzoxy-L-leucinamide (12), we are prompted to present the much simpler synthesis described above in view of the great utility of LA for studies of the enzymes of various tissues and sera. It should be noted that we have usually obtained somewhat faster enzymatic hydrolysis of the compound obtained by direct amidation than with the acetate prepared through the carbobenzoxy intermediate.

manner given by Stahmann, Fruton, and Bergmann (13) with an ethereal solution of leucine methyl ester prepared in the usual way from 19.6 gm. of the hydrochloride and 26 gm. of carbobenzoxyglycyl chloride. After washing and drying the ethereal solution of the product, it was repeatedly concentrated *in vacuo* with dry ether. Yield, 24 gm. of needles on standing with petroleum ether. After recrystallization from ether-petroleum ether, the melting point was 64–66°.

$C_{17}H_{24}O_5N_2$ (336.4). Calculated, N 8.3; found, N 8.3

Carbobenzoxyglycyl-L-leucinamide—The above ester (4 gm.) was amidated in methanol-ammonia in the usual manner. After repeated concentration with methanol, the compound crystallized on standing with a few drops of methanol. Yield, 3.6 gm. After recrystallization from methanol-ether and then from hot water, the melting point was 123–124°.

$C_{16}H_{23}O_4N_3$ (321.3). Calculated, N, 13.1; found, N 13.0, 13.2

Glycyl-L-leucinamide Hydrochloride—The above amide (2.5 gm.) was hydrogenated in the usual manner in the presence of 10 cc. of M HCl, 35 cc. of methanol, and a palladium catalyst. After removal of the catalyst, the solution was concentrated *in vacuo* with ethanol and then with ether. Yield, 1.35 gm. After recrystallization from ethanol-ether, the melting point was 210° (slight browning).

$C_8H_{18}O_2N_3Cl$ (223.7). Calculated, N 18.8; found N 18.8
$[\alpha]_D^{26} = -19.0°$ (5% in water)

L-*Glutamyl*-L-*leucinamide*

Carbobenzoxy-L-glutamyl-L-leucinamide—To a dry ethyl acetate solution of L-leucine methyl ester prepared from 30 gm. of the hydrochloride, there were slowly added 35 gm. of carbobenzoxy-L-glutamic acid anhydride (14). The slightly alkaline solution was allowed to stand at room temperature for 24 hours. It was then washed with dilute hydrochloric acid and with water, dried over Na_2SO_4, and concentrated to a thick oil *in vacuo*. 7 gm. of the oily ester were amidated in methanol-ammonia in the usual manner. After standing at room temperature for 3 days, the solution was concentrated *in vacuo* repeatedly with ether. The residue was extracted into hot ethyl acetate, filtered, and concentrated to dryness. Yield, 3.4 gm.; m.p. 165–169°, after recrystallization from hot water.

$C_{19}H_{27}O_6N_3$ (393.4). Calculated, N 10.68; found, N 10.63

L-*Glutamyl*-L-*leucinamide*—1.0 gm. of the above compound was dissolved in methanol and hydrogenated in the presence of water and acetic acid.

The filtered solution was concentrated repeatedly with absolute ethanol. Yield, 0.5 gm.; m.p. 175–177°.

$C_{11}H_{21}O_4N_3$.　Calculated.　C 51.0, H 8.2, N 16.2
259.3　　　Found.　　" 50.9, " 8.2, " 16.2
$[\alpha]_D^{27} = +6.7°$ (2.1% in water)

L-*Leucyl-L-glutamic Acid*—15 gm. of carbobenzoxy-L-leucine hydrazide (15) were converted to the azide and coupled in ethyl acetate with glutamic acid diethyl ester prepared from 14 gm. of the hydrochloride. After standing at room temperature overnight, the ethyl acetate solution was worked up in the usual manner and concentrated *in vacuo*. The oily product was saponified at room temperature in 10 cc. of methanol and 140 cc. of M NaOH for 90 minutes. It was then acidified to Congo red and evaporated to dryness. The carbobenzoxy-L-leucyl-L-glutamic acid was extracted into ethyl acetate, washed with water, extracted into M sodium bicarbonate, and acidified. The product was again dissolved in ethyl acetate, dried over Na_2SO_4, and concentrated *in vacuo*. The substance was hydrogenated in methanol in the usual manner. Water was added during the hydrogenation to dissolve the crystalline peptide. After removal of the catalyst, the solution was concentrated to dryness with ethanol and then with ether. Yield, 1.1 gm.

$C_{11}H_{20}O_5N_2$.　Calculated.　C 50.7, H 7.7, N 10.8
260.3　　　Found.　　" 50.8, " 7.8, " 10.8
$[\alpha]_D^{24} = +10.5°$ (2% in M HCl)

Fischer (16) found the same rotation with this compound prepared from optically active α-bromoisocapronylglutamic acid.

Carbobenzoxyglycylglycyl-L-leucinamide—2.5 gm. of carbobenzoxyglycylglycyl-L-leucine methyl ester (17) were amidated in methanol-ammonia in the usual way. After repeated concentration to dryness with methanol, the compound crystallized on gentle warming with water. Yield, 1.7 gm.; m.p. 181–182°.

$C_{18}H_{26}O_5N_4$ (378.4).　Calculated, N 14.8; found, N 15.0

SUMMARY

1. Highly purified leucine aminopeptidase of hog intestinal mucosa hydrolyzes glycyl-L-leucinamide, L-glutamyl-L-leucinamide, glycylglycyl-DL-leucylglycine, and L-leucyl-L-glutamic acid in addition to the previously recognized substrates. The hydrolysis of all of these compounds takes place at the carboxyl end of the leucine residue. No endopeptidase action by this enzyme was detected.

2. The concept of aminopeptidase action is revised to include the fact

that the free amino group need not be on the leucine residue which possesses the sensitive peptide bond.

3. The synthesis of a number of new derivatives and peptides of L-leucine is described.

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IODINATION OF HYPOPHYSEAL GROWTH HORMONE*

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It has been shown that if a protein possesses no SH groups, under certain specific conditions it may react with iodine exclusively through the tyrosine residues (1, 2). Since growth hormone as isolated from ox pituitaries contains tyrosine and has no SH groups (3), it would appear interesting to determine whether the tyrosine groups are essential for the growth-promoting action of the hormone after its reaction with iodine. The growth hormone preparations used in the following experiments were isolated by the method previously described (4) and the iodination reactions were carried out at 25°.

Reaction in Acetate Buffer—200 mg. of growth hormone were dissolved in 5 cc. of water with the aid of a few drops of 0.1 N HCl; to the clear solution were added 15 cc. of pH 5.25 acetate buffer of ionic strength 0.10. The suspension was then stirred vigorously while 1 cc. of 0.10 N iodine solution was added drop by drop. The solution was continuously stirred gently. At the end of 20 hours, the solution still had a yellowish tint, indicating the presence of some uncombined iodine. After the excess iodine was removed by a few drops of 0.01 N thiosulfate solution, the solution was first dialyzed against running tap water for 24 hours and then against distilled water for 3 days in a cold room. The iodinated hormone was recovered by lyophilization and the dry material is designated as Preparation A.

In another experiment, conditions were exactly the same as those described above, except that 3.0 cc. of 0.10 N iodine solution were used instead of 1.0 cc. After the removal of the excess iodine with thiosulfate at the end of 20 hours, the solution was thoroughly dialyzed and finally lyophilized. The final product is called Preparation B.

The method of Taurog and Chaikoff (5) was used for iodine determination[1] and the free tyrosine content was determined by the procedure of Lugg (6). Table I presents the analytical results. The theoretical values of iodine were computed from the free tyrosine content by assuming that the lowering of free tyrosine value is due to the formation of diiodotyrosine

[1] We are indebted to J. Wolff for the iodine determination.

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Thus, it may be concluded that tyrosine in the hormone molecule is essential for its growth-promoting activity.

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THE ISOLATION OF PREGNANOL-3(α)-ONE-20, PREGNANEDIOL-3(α),20(β), AND ETIOCHOLANEDIOL-3(α),17(β) FROM THE BILE OF PREGNANT COWS*

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After isolating estrone from the unhydrolyzed bile of pregnant cows (2)' the authors decided to investigate the accompanying neutral fraction, for it appeared likely that progesterone metabolites might be contained therein. The determination of the nature and quantity of such compounds in bile would contribute to our knowledge of progesterone metabolism particularly during pregnancy, and might furnish information as to the degree to which the liver participates in steroid hormone metabolism.

For this purpose, two batches of pooled bile specimens were utilized, Batch A (31.0 liters), from which 20 mg. of crude estrone had been isolated (2), and Batch B (33.0 liters). The extraction and initial fractionation procedures were those previously employed (2). The ether-soluble, neutral fraction of unhydrolyzed bile yielded the following crystalline products but the corresponding fraction obtained from the residual bile after acid hydrolysis did not.

The non-digitonin-precipitable, alcoholic ketonic fraction (Batch A) furnished after chromatography 15 mg. of a crystalline product, m.p. 147–148°. It was purified and its identity as pregnanol-3(α)-one-20 established by determination of the melting point before and after admixture with pregnanol-3(α)-one-20, carbon and hydrogen analysis, determination of the specific optical rotation, and the preparation of an acetyl derivative which gave the expected carbon and hydrogen values on analysis and which did not depress the melting point after admixture with pregnanol-3(α)-one-20,3-acetate.

A search was made for progesterone in the non-alcoholic ketonic fraction (Batch A). Examination of this material after chromatography revealed several fractions with intense absorption at 240 mμ. These fractions were combined and induced to crystallize, thereby furnishing Compound X, m.p. 215–218°. It exhibited an ultraviolet absorption spectrum almost identical

with that of progesterone (see Fig. 1) but comparison of the melting points and distribution coefficients (see Table II) clearly established its non-identity with progesterone as well as with certain other non-alcoholic, α,β-unsaturated ketones. Compound X was isolated in an amount insufficient for carbon and hydrogen analysis, but as much as 13 mg. may have been contained originally in the non-alcoholic ketonic fraction. This
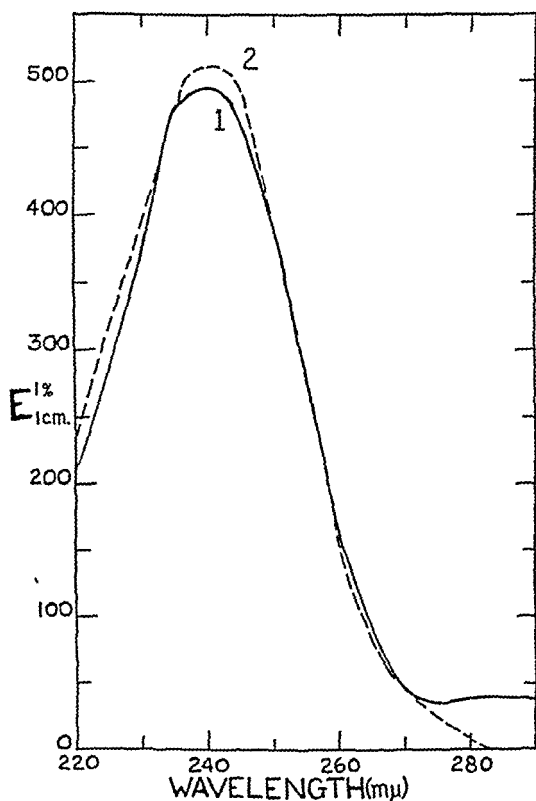
FIG. 1. Ultraviolet absorption spectra of Compound X (Curve 1) and of progesterone (Curve 2). Solvent, absolute ethanol.

figure is based on the relative extinction coefficients of Compound X and of the fraction from which it was derived.

The non-ketonic fraction (Batch A) yielded 22 mg. of crude Compound Y and 26 mg. of crude Compound Z. These compounds, in amounts of 26 and 55 mg. respectively, were also isolated from the non-digitonin-precipitable, non-ketonic fraction of Batch B. The products were purified and their identities established as follows. Compound Y, m.p. 235–236°, gave no melting point depression on admixture with pregnanediol-3(α),20(β) prepared from either pregnanol-3(α)-one-20 or progesterone. It was

further identified by carbon and hydrogen analysis, specific optical rotation, and the preparation of a diacetyl derivative which did not depress the melting point on admixture with pregnanediol-3(α),20(β),3,20-diacetate. Compound Z, m.p. 228.5–229°, did not depress the melting point on admixture with etiocholanediol-3(α),17(β), exhibited practically the same specific optical rotation, and gave the expected carbon and hydrogen values on analysis. Furthermore, Compound Z yielded on chromic acid oxidation a product which gave no melting point depression on admixture with etiocholanedione-3,17, exhibited the correct specific optical rotation, and gave the expected carbon and hydrogen values on analysis.

<div align="center">EXPERIMENTAL[1]</div>

Collection and Extraction of Bile—Gallbladder bile was freshly collected from slaughtered cows estimated to be at least 5 months pregnant.[2] The bile was refrigerated and extracted within 24 to 48 hours subsequent to collection. Two batches of bile were utilized to obtain the neutral fraction of *unhydrolyzed* bile: Batch A (31.0 liters) was the same collection of bile from which estrone had been isolated in this laboratory (2); Batch B (33.0 liters) was worked up[3] in order to procure additional non-ketonic, neutral products.

Procedures have already been described (2) for obtaining the neutral material; it is ether-soluble and constitutes the 90 per cent methanol phase after repeated partitioning with petroleum ether.

Fractionation of Neutral Material of Unhydrolyzed Bile—Separation into ketonic and non-ketonic moieties was achieved with the aid of Girard's Reagent T(3). The ketones on treatment with succinic anhydride in pyridine (4) were resolved into alcoholic and non-alcoholic fractions. Digitonides were precipitated by treating the alcoholic ketones and the non-ketones respectively with hot 70 per cent methanol containing 1 per cent digitonin; the digitonides were split in the usual way with pyridine and dry ether (5).

Isolation of Pregnanol-3(α)-one-20—The non-digitonin-precipitable, alcoholic ketonic fraction (218 mg. from Batch A) was dissolved in 1.5 ml. of

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[1] All melting points reported are corrected except where otherwise indicated.

[2] We are indebted to Dr. C. E. Mootz of the United States Department of Agriculture, Philadelphia, for arranging a veterinarian-supervised collection of bile. Dr. Irwin Rothman was of great assistance in judging the approximate stage of pregnancy by examination of the fetuses.

[3] Batch B was worked up in a slightly different way. The bile prior to extraction with butanol was made acid to Congo red with concentrated HCl. This resulted in a more rapid separation of the two phases. The butanol extracts were washed with a little water, neutralized with a few drops of concentrated NH_4OH, and evaporated *in vacuo*, etc.

benzene and 1.4 ml. of petroleum ether (b.p. 35–45°) added. The solution was passed through a narrow column containing 4 gm. of alumina[4] previously wetted with the same solvent mixture. The adsorbed material was successively eluted with benzene-petroleum ether, benzene, benzene-ether, ether, ether-methanol, and methanol. The benzene (25 to 75 per cent)-petroleum ether eluates (a total volume of 725 ml. of eluant was employed) contained 25 mg. of nicely crystalline material. On recrystallization from aqueous methanol it gave 15 mg. of needles, m.p. 147–148°. Further recrystallization from the same solvent mixture yielded a product melting sharply at 148–148.5°, $[\alpha]_D^{26} = +108.5° \pm 1°$ (9.23 mg. in 1.23 ml. of absolute ethanol solution). The material was recovered and recrystallized for analysis.

$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.35, H 10.64

There was no depression of the melting point on admixture with an authentic specimen[5] of pregnanol-3(α)-one-20, m.p. 148.5–149°.

Following treatment of 8.6 mg. of the above material, m.p. 147–148°, with acetic anhydride and pyridine for 24 hours at room temperature, a product was obtained which on repeated recrystallization from aqueous methanol gave 4 mg. of plates, m.p. 97–98°.

$C_{23}H_{36}O_3$. Calculated, C 76.62, H 10.06; found, C 76.75, H 10.20

The melting point was not depressed on admixture of this product with pregnanol-3(α)-one-20,3-acetate, m.p. 99–100°, prepared under identical conditions.

The digitonin-precipitable alcoholic ketonic fraction derived from Batch A weighed 44 mg. It was chromatographed, thereby yielding 1 mg. of a slightly impure crystalline product, m.p. 184–186°, which was not identified.

Isolation of Compound X—The non-alcoholic ketonic fraction (152 mg. from Batch A) was dissolved in 3 ml. of benzene and 1.8 ml. of petroleum ether added. The material was adsorbed on a narrow column containing 4 gm. of alumina and eluted successively with benzene-petroleum ether, benzene, benzene-ether, ether, ether-methanol, and methanol. The material (41 mg.) eluted by 125 ml. of ether (25 to 75 per cent)-benzene exhibited a maximum absorption at 240 mμ; $E_{1\,cm.}^{1\%} = 155$ (in absolute ethanol). Crystallization from ether yielded 5 mg. of a crude product,

[4] The alumina employed throughout this study is described as Harshaw, plain activated. Prior to use, it was suspended in glacial acetic acid, filtered, washed with copious amounts of distilled water, dried at 150° for several hours, and finally placed in a tightly sealed container.

[5] Kindly furnished by Dr. Seymour Lieberman. Butenandt and Müller (6) reported pregnanol-3(α)-one-20, m.p. 148–149° (uncorrected), $[\alpha]_D^{19} = +113.9° \pm 1.2°$ (in absolute ethanol); acetyl derivative, m.p. 99° (uncorrected).

m.p. 206–212°; repeated recrystallization from the same solvent gave 1.0 mg., m.p. 215–218°. The latter material is referred to as Compound X. Its ultraviolet absorption spectrum is almost identical with that of progesterone (see Fig. 1); $E_{1\,cm.}^{1\%} = 494$ and 509 at 240 mμ for the two compounds respectively. However, Compound X is not identical with progesterone nor with certain other non-alcoholic, α,β-unsaturated ketones because of marked differences in the melting points and distribution coefficients of the respective compounds (see Table I).

Chromatography of the mother liquors (40 mg.) of Compound X did not result in any substantial degree of purification. In fact, an appreciable

TABLE I

Distribution Coefficients: Compound X and Other Non-Alcoholic α,β-Unsaturated Ketones

Substance	M.p.	Distribution coefficient,* petroleum ether-70 per cent methanol
	°C.	
Compound X	215–218	0.05
Progesterone	127–128	0.33
Δ-4-Cholestenone-3	81–82	3.07
Δ-4-Androstenedione-3,17	173–174	0.07

* Determined by distributing approximately 1 mg. of the substance at room temperature between 50 ml. portions of petroleum ether (previously washed with concentrated H_2SO_4, then water, and distilled at 35–45°) and of 70 per cent methanol; the solvents were mutually saturated prior to use. The residues obtained from each phase were taken up in absolute ethanol and the densities determined at 240 mμ. The ratio of the densities gave the distribution coefficient.

amount of material absorbing at 240 mμ failed to be recovered in the process. Counter-current distribution of the remaining material seemed to be a more effective means of purification; an additional 1 mg., m.p. 213–217°, was thereby obtained.

Isolation of Pregnanediol-3(α),20(β) (Compound Y) and of Etiocholanediol-3(α),17(β) (Compound Z)—The non-ketonic fraction (801 mg. from Batch A) was treated with ethyl acetate at room temperature, thereby furnishing 183 mg. of crystalline material, m.p. 203–217°. Recrystallization from the same solvent yielded 142 mg. of a product, m.p. 200–208°, which although nicely crystalline, proved to be a complex mixture. Details of the chromatographic analysis are not furnished in this instance but are described below for the non-ketonic fraction obtained from Batch B. Chromatography was more effectively carried out in the latter instance.

The non-ketonic fraction (Batch A) furnished 22 mg. of impure Co

pound Y, m.p. 231–235°, which was purified by chromatography. There were finally obtained 5 mg., m.p. 234–236°, $[\alpha]_D^{31} = +18° \pm 4°$ (4.77 mg. in 1.23 ml. of dioxan solution); it crystallized in needles from ethyl acetate and in rectangular plates from ethanol.

The non-ketonic fraction (Batch A) also furnished 26 mg. of somewhat impure Compound Z, m.p. 227–229°, $[\alpha]_D^{29} = -9°$ (in dioxan). Repeated recrystallization from ethyl acetate gave 10 mg. of needles, m.p., 229–229.5°.

$C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 78.15, H 11.07

The non-ketonic fraction (Batch B) after the removal of digitonin-precipitable material (13 mg. of oil) weighed 856 mg. When treated with ethyl acetate, it formed a gel. The material was consequently recovered and

TABLE II

Chromatographic Analysis of Non-Ketonic Fraction (Non-Digitonin-Precipitable, Batch B)

Fraction No.	Eluant		Eluate	
	Volume	Composition	Weight	Description or compounds isolated
	ml.		*mg.*	
1– 5	115	Benzene	327	Semicrystalline; Compounds Y and Z
6–12	310	"	111	Compound Z
13–15	100	Benzene-ether (25%)	33	" "
16–22	200	" (50–100%)	61	Colorless glass
23–24	50	Ether-methanol (1%)	38	" "
25–26	50	" (5%)	131	Unidentified crystals, 4 mg.; m.p. 253–254°
27–34	215	" (5–50%)	79	Oil
35–37	50	Methanol	5	"

taken up in 0.5 ml. of acetone and 3 ml. of benzene were added. The solution was passed through a column (90 × 20 mm.) containing 15 gm. of alumina previously wetted with benzene. A condensed version of the chromatographic analysis is given in Table II.

Fractions 1 to 5 (total 327 mg., see Table II) were combined, which contained 46 mg. of benzene-insoluble material which, on treatment with ethanol, yielded 27 mg. of slightly impure Compound Y, m.p. 233–236°. Repeated recrystallization from the same solvent gave 16 mg. of rectangular plates, m.p. 235–236°, $[\alpha]_D^{25} = +19° \pm 5°$ (10.8 mg. in 2.00 ml. of absolute ethanol solution).

$C_{21}H_{36}O_2$. Calculated, C 78.69, H 11.33; found, C 78.73, H 11.25

It gave no melting point depression on admixture with pregnanediol-3(α),20(β), m.p. 235° (rectangular plates from ethanol), which had been

prepared in this laboratory by hydrogenation of pregnanol-3(α)-one-20 in glacial acetic acid containing platinum oxide. Comparison of Compound Y was likewise made with a specimen of pregnanediol-3(α),20(β), m.p. 235°, $[\alpha]_D^{27} = +18° \pm 4°$ (6.9 mg. in 1.23 ml. of absolute ethanol solution), which had been prepared in this laboratory from progesterone under the same conditions of hydrogenation as above. A specimen[6] of slightly impure pregnanediol-3(α),20(β), m.p. 234–234.5°, which was kindly furnished by Dr. Miescher, did not depress the melting point on admixture with our synthetic preparation. On the other hand, admixture of Compound Y, m.p. 235–236°, with pregnanediol-3(α),20(α), m.p. 238–239°, gave a melting point depression of 22–26°.

Treatment of 11 mg. of Compound Y in pyridine and acetic anhydride at room temperature for 24 hours gave 13 mg. of a product which could not be crystallized from methanol. It crystallized, however, from pentane on careful chilling in an alcohol-solid CO_2 bath; further recrystallization from the same solvent gave 5 mg. of needles, m.p. 109–109.5°. It did not depress the melting point on admixture with an authentic specimen[6] of pregnanediol-3(α),20(β),3,20-diacetate, m.p. 110–110.5°.

A small amount (3 mg.) of Compound Z, m.p. 225–227°, was obtained on chromatographic analysis of the benzene-soluble material (281 mg.) contained in Fractions 1 to 5 (Table II). Fractions 6 to 15 (Table II) yielded more of this compound on treatment with ethanol, 52 mg. of needles, m.p. 223–227°, being obtained. Repeated recrystallization from the same solvent gave 30 mg. of pure Compound Z, m.p. 228.5–229°, $[\alpha]_D^{25} = -1.6° \pm 1.4°$ (30.1 mg. in 3.00 ml. of absolute ethanol solution).

$C_{19}H_{32}O_2$.   Calculated, C 78.03, H 11.03; found, C 78.12, H 11.05

It did not depress the melting point on admixture with a specimen[7] of etiocholanediol-3(α),17(β), m.p. 226–227°, $[\alpha]_D^{28} = 0 \pm 1.8°$ (in ethanol). Acetyl and benzoyl derivatives of Compound Z were prepared but these failed to crystallize.

To a solution of 12.0 mg. of Compound Z, m.p. 225.5–227°, in 1 ml. of glacial acetic acid was added 0.8 ml. of 1 per cent CrO_3 in the same solvent.

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[6] Dr. K. Miescher gave as the melting points of the specimens of pregnanediol-3(α),20(β) and its diacetyl derivative 240–241° and 113–115° respectively. These observations (cf. (7)) were apparently made under the microscope according to Kofler, whereas in this laboratory melting point determinations are performed on the specimen in a capillary tube immersed in an oil bath containing a long stem standardized thermometer.

[7] This specimen was kindly furnished by Dr. H. L. Mason who with Dr. J. J. Schneider obtained it on incubating etiocholanol-3(α)-one-17 with liver slices (8). Dr. Mason in a private communication stated that he likewise was unsuccessful in preparing a crystalline acetate or benzoate.

The reaction mixture was allowed to stand at room temperature for 24 hours and, after adding a little methanol, was poured into water and extracted with ether. The ether solution was washed successively with dilute HCl, NaHCO$_3$ solution, and finally with water. Evaporation of the ether yielded 13 mg. of material which gave, on repeated recrystallization from ether-pentane, 4 mg. of plates, m.p. 132–133°, $[\alpha]_D^{24} = +123° \pm 8°$ (3.13 mg. in 1.23 ml. of absolute ethanol solution). The material was recovered for analysis.

$C_{19}H_{28}O_2$. Calculated, C 79.13, H 9.78; found, C 79.44, H 9.90

It gave no melting point depression on admixture with a specimen[8] of etiocholanedione-3,17, m.p. 132°. It gave a melting point depression of 20–22° on admixture with a specimen[9] of androstanedione-3,17, m.p. 131.5–132°.

DISCUSSION

The isolation from bile of pregnanol-3(α)-one-20, a known (10) metabolite of progesterone, implicates the liver in the metabolism of progesterone. This view is supported by a substantial amount of indirect evidence (e.g., (11, 12)) accumulated in recent years which indicates that the liver is an important if not major site of progesterone inactivation. Pregnanol-3(α)-one-20 is a characteristic constituent of the urine of pregnancy (13–15) but has hitherto not been sought for in bile.

The accompanying product, pregnanediol-3(α),20(β), which is excreted in bile but curiously enough not in urine, may be considered to be a metabolite of pregnanol-3(α)-one-20 inasmuch as it can readily be prepared by catalytic hydrogenation of the latter substance. Its stereoisomer, pregnanediol-3(α),20(α), which is definitely known[10] to be a metabolite of progesterone and is a major urinary steroid of the pregnant cow (and of many but not all species), is conspicuous by its absence from the bile of this species. However, in a study (17) in which massive doses of pregnenol-3(β)-one-20 were administered orally to a postmenopausal woman, minute amounts of pregnanediol-3(α),20(α) were obtained from the bile. Whether pregnanediol-3(α),20(α) is a normal constituent of the bile of pregnant women remains to be investigated. Another stereoisomer of pregnanediol occurring in bile but not in urine is allopregnanediol-3(β),20(β); this substance was previously obtained from ox bile (18) but failed to be isolated in this instance from cow bile.

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[8] This specimen was kindly furnished by Dr. Erwin Schwenk. Ercoli and Mamoli (9) reported etiocholanedione-3,17, m.p. 131–132°, $[\alpha]_D^{18} = +113°$ (ethanol).

[9] Kindly furnished by Dr. Seymour Lieberman.

Although products related to progesterone metabolism were obtained from bile, progesterone itself was not isolated. However, another substance (Compound X) which, like progesterone, appears to be an α,β-unsaturated, non-alcoholic ketone, was isolated. Unfortunately, not enough material was available for further structural elucidation. In this connection, it might be well to point out that progesterone can barely be detected in pregnancy urine (19) although its reduction products are abundantly present therein; the progesterone level in blood (20) is likewise estimated to be very low.

Etiocholanediol-3(α),17(β) has not been isolated from urine although its 17-epimer, etiocholanediol-3(α),17(α), is known (20–23) to be present. Both compounds are formed following the incubation of etiocholanol-3(α)-one-17 with surviving rabbit liver slices (8). The same reactions probably occur *in vivo* in view of the isolation of etiocholanediol-3(α),17(β) from bile; the *in vivo* formation of etiocholanediol-3(α),17(α) from dehydroisoandrosterone has recently (23) been demonstrated.

The presence in pregnancy bile of a C_{19} steroid (which incidentally is probably devoid of androgenic activity) is not altogether surprising since other compounds in this category, such as dehydroisoandrosterone and androsterone, are found (24, 25) in pregnancy urine. It is remarkable, however, that the ratio of the quantities of C_{19} steroid to C_{21} steroid (progesterone reduction products) is so high in the former case. Pertinent to this observation is the recent finding (26) that the pregnant cow excretes considerable quantities of androgenic material in the feces. It is of interest also that the neutral compounds of cow bile, including the estrogens (2), are excreted as such, whereas in the urine of most species these or related products exist predominantly in a conjugated form.

SUMMARY

Pregnanol-3(α)-one-20, pregnanediol-3(α),20(β), and etiocholanediol-3(α),17(β) were obtained in amounts ranging from approximately 0.5 to 2 mg. per liter from gallbladder bile of cows in an advanced stage of pregnancy. An unidentified substance (Compound X) was also isolated but in an amount insufficient for analysis; it is not identical with progesterone although it possesses some of its chemical features. The foregoing products were found in the neutral fraction of unhydrolyzed bile; the corresponding fraction of the residual bile after acid hydrolysis failed to yield any crystalline material.

A discussion has been presented which deals with the significance of these

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[10] For a discussion of the intermediary metabolism of progesterone (and other steroid sex hormones) see Pincus and Pearlman (16).

findings, particularly as it pertains to the rôle of the liver in steroid hormone metabolism.

The authors are indebted to Mr. James Rigas for the microanalyses.

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THE KINETICS OF THE OXIDATION OF BENZOIC ACID BY CERTAIN MYCOBACTERIA

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Benzoic acid is oxidized by certain *Mycobacteria* (1). They do not oxidize it completely to carbon dioxide and water; the oxygen uptake usually stops when 5 atoms of oxygen are utilized for each molecule of benzoic acid. The enzymes concerned are adaptive, and are rapidly formed when small amounts of benzoic acid are added to suspensions of the bacteria (2). Benzoic acid may be considered somewhat toxic to the bacteria, because, although small amounts stimulate enzyme formation, larger amounts slow down or inhibit the process, possibly by interfering with reactions providing the energy for the enzyme synthesis. Furthermore, as shown below, the enzyme once formed disappears fairly rapidly in the absence of benzoic acid. Streptomycin prevents the formation of these adaptive enzymes, and thus inhibits oxidation of benzoic acid (2). The rate of oxygen uptake has been carefully measured and the following is an analysis of the kinetics of the reactions involved. Data were obtained from experiments done with *Mycobacterium tuberculosis* BCG 8240. The technique has already been described (1, 2).

Oxidation of benzoic acid by *Mycobacteria* proceeds by a number of stages. The substances formed, for the most part, have not been isolated, and will be referred to by letters. Benzoic acid (A) is first hydrated (*cf.* (3)) to form B. B takes up 1 atom of oxygen and becomes C. C, in turn, takes up a second atom of oxygen to become D, and so on through E, F, and G, until 5 atoms of oxygen have been taken up for each molecule of the original benzoic acid. The time course of each of these reactions, since there is no change in the concentrations of either water or oxygen, will probably be monomolecular. Using small letters to mean "concentration of," we may express these rates as follows:

$$\frac{da}{dt} = -k_1 a$$

$$\frac{db}{dt} = k_1 a - k_2 b$$

$$\frac{dc}{dt} = k_2 b - k_3 c$$

$$\dots\dots\dots\dots\dots\dots\dots$$

$$\frac{df}{dt} = k_5 e - k_6 f$$

Because we do not know how G is further transformed by the bacteria, except that it is not oxidized, it is difficult to write an equation for it; later it will be seen that it is unnecessary to do so. In each case the velocity
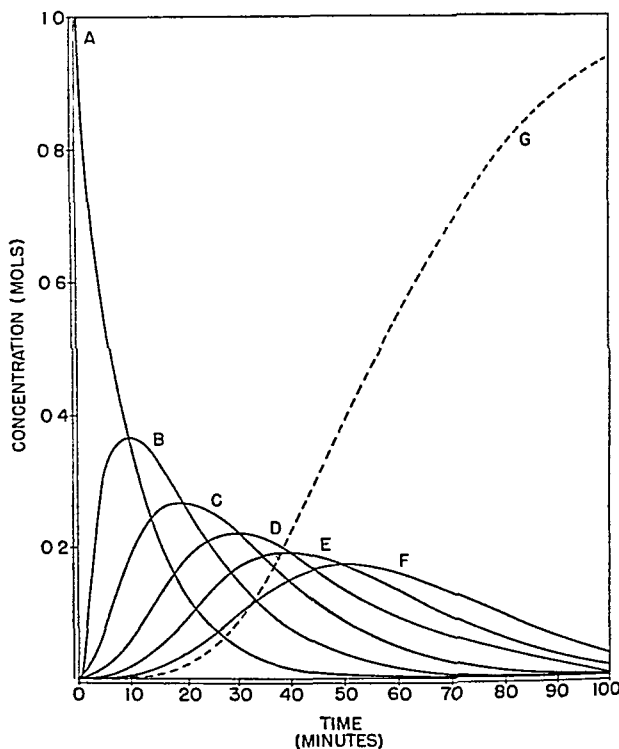
FIG. 1. The different curves give the amounts of benzoic acid (A) and substances B, C, D, E, and F at the times indicated. The original amount of A is taken as 1.0 mole and the k values are put equal to 0.1 each.

constant is proportional to the amount of the corresponding enzyme present in any particular experiment; an increase in the constant implies an increase in the amount of enzyme.

Equations similar to these have been integrated by Bateman (4). We may, however, begin by making the simplifying assumption that all the constants, k_1, k_2, etc., are equal. Integration then leads to a series of equations giving the amounts of each substance present at various times. The general solution giving the amount of the Nth substance is

$$n = e^{-kt} \frac{c_1}{(N-1)!} (kt)^{N-1} + \frac{c_2}{(N-2)!} (kt)^{N-2} + \dots c_{N+1}$$

The constants of integration, c_1, c_2, etc., may be evaluated by setting the condition that at zero time there is present in the mixture a_0 of A, and none of the other substances, B, C, etc. Then c_1 becomes a_0, and the other constants, c_2, c_3, etc., become zero. Fig. 1 shows the amounts of these substances present at various times when a_0 is given the value 1, and all the k values are put equal to 0.1.

To compute the oxygen consumption we proceed as follows. At the time t, each molecule of C that is present has taken up 1 atom of oxygen, each molecule of D has taken up 2 atoms, etc. G presents a slight difficulty since it may have been further transformed; we must therefore say that 5
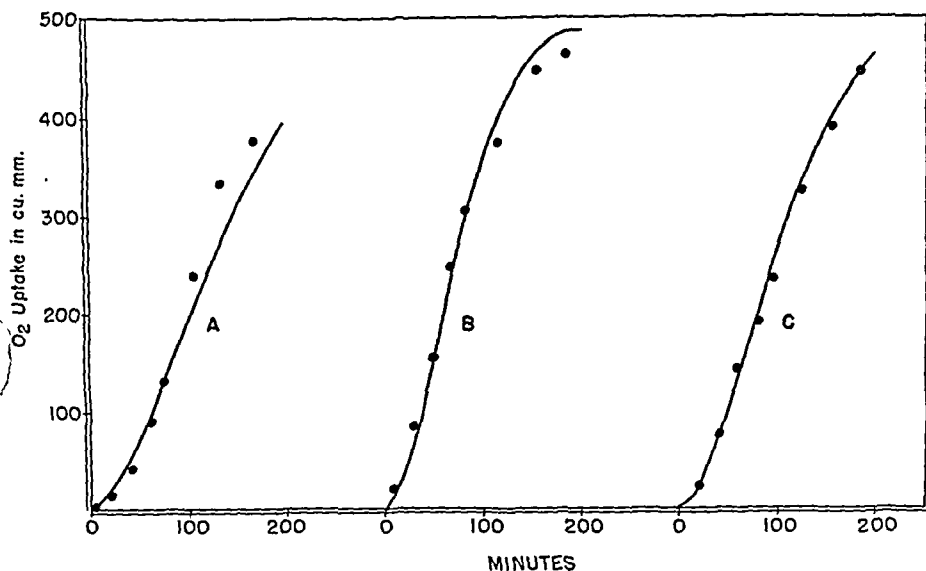
Fig. 2. Oxidation of 1.0 mg. of benzoic acid by *Mycobacterium tuberculosis* BCG 8240 preincubated for 90 minutes (Curve A) without benzoic acid; Curve B and C with 0.05 mg. of benzoic acid. 1.0 mg. of benzoic acid was added to each after preincubation, and in addition 10 γ of streptomycin (Curve C). The points are experimental, and the curves are theoretical; computed for Curve A $k = 0.028$, Curve B $k = 0.050$, and Curve C $k = 0.038$.

atoms of oxygen have been taken up by all of the original benzoic acid molecules that have reached this stage or gone beyond it. But all molecules not in the forms A, B, C, D, E, or F have reached this stage or gone beyond it, and their concentration must therefore be $a_0 - (a + b + c + d + e + f)$. The total oxygen taken up, X, will then be given by

$$X = c + 2d + 3e + 4f + 5(a_0 - (a + b + c + d + e + f))$$

which simplifies to

$$X = 5a_0 - (5a + 5b + 4c + 3d + 2e + f)$$

When appropriate values for the constants a_0 and k are chosen, oxygen consumption at various times may be computed; the smooth curves in Fig. 2 were constructed from such computations. Experimental data, adjusted so that a_0 corresponds to exactly 500 c.mm. of oxygen, were plotted on these theoretical curves. It was then seen that the experimental points, although forming a curve of precisely the same shape as the theoretical, all lay a short distance to the right of it. When shifted 10 to 15
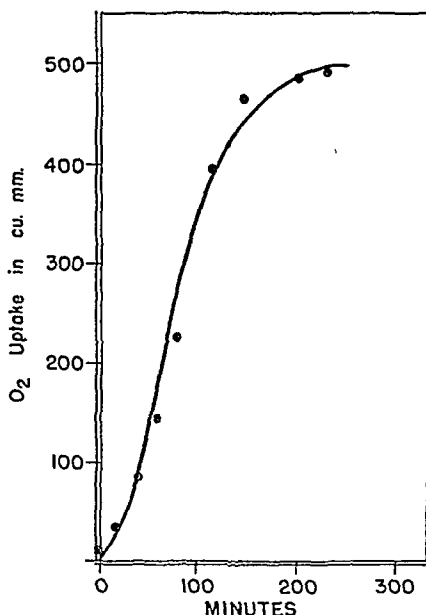
FIG. 3. A smooth curve is made up on the assumption that the velocities of the oxidations of E to F and of F to G are increased so that E and F do not accumulate in any significant amount. The points are experimental.

minutes to the left, the fit was excellent, as shown in Fig. 2. This shift implies a latent period before any great amount of oxidation occurs; this time interval is presumably occupied by formation of enzyme. It will be discussed further below.

Of five experiments analyzed, three yielded results that fitted such curves; two did not. In these cases the assumption was then made that the velocities of the oxidations of E to F and of F to G were increased to such an extent that these substances, F and G, never accumulated in any significant amount. New curves based on this additional assumption were constructed, and experimental results were found to fit these in a satisfactory manner (Fig. 3).

Table I gives values for the k constants and the lengths of the latent periods for one set of experiments. For convenience a set was chosen in which the k values were equal in each experiment. It will be seen that the latent period is longest when no benzoic acid was used for preincubation; it disappears completely after 90 minutes preincubation with 0.2 mg. of benzoic acid. These facts may be explained on the assumption already mentioned, that benzoic acid has a double action. In very low concentrations it stimulates the formation of the oxidizing enzymes, while in high

TABLE I

Values for k Constants and Lengths of Latent Periods

Experiment No.	Benzoic acid used for preincubation	Without streptomycin		With streptomycin
		Latent period	k	k
	mg.	*min.*		
13-14	None	18	0.028	0.017
16-17	0.05	10	0.050	0.038
21-22	0.10	4	0.050	0.050
24-25	0.20	0	0.050	0.050

TABLE II

Demonstration of Disappearance of Enzyme

In this experiment k_5 and k_6 were larger than the others; the values given are for k_1 to k_4 which were equal.

Time of preincubation with 10 γ benzoic acid	k
min.	
0	0.43
60	0.67
90	0.67
120	0.50
240	0.36

concentrations its toxic action tends to depress the formation of enzymes. Thus when no benzoic acid was added during preincubation no enzymes were formed, the latent period was long, and the total enzyme formed was least, as shown by the value for k. With very small amounts, some enzyme was formed, but the addition of the large amount of benzoic acid for the experiment induced some further formation. The latent period was shorter. With larger amounts the maximum amount of enzyme is formed, the k reaches a maximum, and the latent period vanishes. Enzyme already formed is apparently not interfered with.

Disappearance of enzyme was demonstrated in the following way. Bacteria was preincubated with 0.01 mg. of benzoic acid for various lengths

of time. As long as benzoic acid was present the enzymes increased to a maximum; after it was exhausted they gradually decreased. Data are given in Table II.

The effect of streptomycin is to decrease the velocities of all the reactions equally; there is no specific effect on any particular stage. This is true whether all the k values are equal, as in Table I, or whether they are not, as in other experiments.

SUMMARY

The kinetics of the oxidation of benzoic acid by *Mycobacterium tuberculosis* BCG 8240 have been analyzed, and six consecutive monomolecular reactions have been postulated. In most cases the velocity constants for these reactions were the same; in other cases those for reactions 5 and 6 were considerably greater, due to a relative increase in the amounts of the adaptive enzymes which catalyze these processes. Oxidation begins with a latent period during which adaptive enzymes are formed. If the bacteria are previously treated with small amounts of benzoic acid, the latent period becomes shorter or vanishes, and the constants become greater up to a maximum, indicating the presence of increased amounts of enzyme. Enzymes gradually disappear in the absence of benzoic acid.

The effect of streptomycin is to depress equally all stages of oxidation.

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THE PRODUCTS OF PROTEOLYSIS OF SOME PURIFIED PROTEINS*

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Recent studies on the digestion of proteins by the proteolytic enzymes of the gastrointestinal tract have resulted in a considerable revision of our concepts on how these enzymes act.

Tiselius and Eriksson-Quensel (1) studied the mechanism of the peptic digestion of crystalline egg albumin by electrophoretic examination of the products of proteolysis. They observed that, as digestion proceeded, the average size of the peptides formed remained constant. From these results, they postulated that each protein molecule is rapidly degraded to its ultimate products without the intervening production of larger units. They have called this an "all or none" mechanism of proteolysis. Haugaard and Roberts (2) reached similar conclusions from experiments on the digestion of β-lactoglobulin by crystalline pepsin. They reported that during digestion the increase in terminal amino nitrogen (determined by the nitrous acid manometric technique of Van Slyke (3)) increased lineally with the total non-protein nitrogen. A similar study was made by Winnick (4) on the digestion of casein by chymotrypsin, trypsin, pepsin, ficin, and papain. His data were consistent with those of the above workers.

We have extended these observations in the present paper, employing for our studies trypsin acting on crystalline bovine serum albumin and purified γ-globulin, and pepsin on crystalline bovine serum albumin, purified bovine fibrin, and twice recrystallized egg albumin. The results to be presented also favor a rapid or immediate degradation of the protein molecules to peptides of characteristic size, with the liberation of little or no free amino acid nitrogen.

EXPERIMENTAL

Enzymes and Substrates—Solutions of crystalline trypsin were made in 0.05 M phosphate buffer at pH 7.8. The pepsin was suspended in water and dialyzed free of $MgSO_4$ before each experiment.

The substrates were dissolved in 0.05 M phosphate buffer, pH 7.8, for the trypsin experiments and in HCl at pH 1.8 for peptic digestion.

We are indebted to the Department of Physical Chemistry of the Harvard Medical School for our supply of protein substrates.[1]   Fibrin was prepared by clotting the fibrinogen in Fraction I of bovine serum (7) with thrombin.

For experiments with fibrin, the substrate was suspended in HCl or homogenized in HCl at pH 1.8.

Procedure—The solutions of protein were incubated with the enzyme at 38° in the amounts indicated in Tables I to V.   Digestion was carried out in 50 cc. Erlenmeyer flasks, and suitable aliquots were withdrawn at given time intervals for analysis.   In the experiments with pepsin, in which the digestion products were separated by dialysis (see below), the substrates were incubated in a cellophane dialyzing sac and dialyzed against an equal volume of HCl at pH 1.8.

Method of Analysis—Three different procedures were employed for the removal of undigested protein from the samples to be analyzed.

In the first, the protein was precipitated with an equal volume of 10 per cent trichloroacetic acid or 1 per cent picric acid.   The solution was filtered, and in the case of trichloroacetic acid, the filtrates were acidified to pH 1 with HCl and extracted with ether.   This extraction was necessary, as the trichloroacetic acid interfered with the subsequent analysis; it was omitted on picric acid filtrates.   All experiments with trypsin were carried out by this procedure.

In the second method, digestion was carried out in a cellophane sac suspended in HCl at pH 1.8.   Analyses were then made on aliquots of the dialysates.

Finally, experiments were carried out with a rocker-perfusion apparatus originally designed for cultivation of malarial parasites (8).   In this method, the products of proteolysis were continuously removed by dialysis against a stream of aqueous HCl, pH 1.8, passing through a cellophane coil immersed in the digestion mixture.

Aliquots of the protein-free filtrates and dialysates, prepared as described above, were analyzed for terminal amino nitrogen by the Van Slyke nitrous acid method (3) and for free amino acid nitrogen by the ninhydrin method (9).   The amino acids bound in peptides were determined by the ninhydrin method after hydrolysis.   This is referred to in Tables I to V as total amino nitrogen.

The average number of amino acid residues per peptide molecule was determined by the ratio of the "total" to the "terminal" amino nitrogen. A correction was made for free amino acid nitrogen when significant. All values are expressed as mg. of NH_2-N or COOH-N released in the total reaction mixture.

Hydrolysis—The peptides were hydrolyzed in 6 N HCl at 110° for 12 to 18 hours. The hydrolysates were concentrated to dryness *in vacuo* over sodium hydroxide pellets to remove excess HCl, and the residues were made up to a known volume for analysis.

TABLE I

Digestion of Bovine Serum Albumin and Human γ-Globulin by Crystalline Trypsin at pH 7.8

Experiment 1, 100 cc. of 2 per cent serum albumin + 20 mg. of trypsin; Experiment 2, 100 cc. of 2 per cent serum albumin + 50 mg. of trypsin; Experiment 3, 50 cc. of 1 per cent γ-globulin + 20 mg. of trypsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH_2-N)	Total amino nitrogen (COOH-N)	$\dfrac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
	hrs.	*mg.*	*mg.*	*mg.*	
1	4	Not analyzed	5.1	14	2.7
	8	" "	8.4	22	2.6
	24	0.6	13.5	42	3.2
	48	1.4	23.5	60	2.7
2	34		13.5	35	2.6
	48		13.8	36	2.7
	56		13.8		
3	4		0.75·	2.0	2.7
	9		1.30	3.5	2.7
	24	<1% terminal amino-N	1.75	5.6	3.2
	48	<1% " "	2.34	8.5	3.6

Results

Digestion of Serum Albumin and γ-Globulin with Trypsin—The results of experiments with trypsin are given in Table I. Increasing the concentration of trypsin from 20 to 50 mg. did not accelerate digestion. It is evident that the digestion of γ-globulin by trypsin proceeds very slowly, but it is of interest that there is no evidence for the presence of larger peptides in the protein-free filtrate, although the first analyses must represent the early stages of digestion. For serum albumin, the average number of amino acid residues per peptide molecule is about 2.7; for γ-globulin, the values ranged from 2.7 to 3.6.

Digestion of Crystalline Egg Albumin and Serum Albumin with Pepsin—
Owing to the slow rate of digestion of native proteins by trypsin, the follow‑
ing experiments were carried out with crystalline pepsin. Table II gives
the results of the digestion of egg albumin and serum albumin by this
enzyme.

In Experiment 1, the undigested egg albumin was precipitated with 10 per
cent trichloroacetic acid as previously described. In Experiment 2, the
undigested serum albumin was precipitated with picric acid. It is evident
from Experiment 1 that, although after 4 hours the digestion was nearing
completion, the subsequent 20 hour incubation of the peptides with the
enzyme resulted in no further degradation of the peptide molecules.

TABLE II

Digestion of Egg Albumin and Bovine Serum Albumin by Crystalline Pepsin at pH 1.8

Experiment 1, 100 cc. of 1 per cent egg albumin + 10 mg. of pepsin; Experiment 2,
15 cc. of 3.3 per cent serum albumin + 10 mg. of pepsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH$_2$-N)	Total amino nitrogen (COOH-N)	$\frac{COOH-N}{NH_2-N}$, average No. of amino acid residues per peptide molecule
	hrs.	*mg.*	*mg.*	*mg.*	
1	4		10.7	72.0	6.7
	6		11.4		
	24	0.32	13.2	93.0	7.0
2	0.25		1.2	4.4	3.7
	4	<1% terminal amino N	3.2	10.1	3.2

These experiments on egg albumin, in which the value of seven amino
acid residues per peptide molecule was obtained, are in good agreement with
the value obtained by Tiselius and Eriksson‑Quensel who found the average
molecular weight of the peptides to be 1080 from diffusion coefficient
measurements. For serum albumin, the average ratio of the total peptide
bond amino nitrogen to terminal amino nitrogen was about 3.5, or about
half that of egg albumin.

Digestion of Purified Fibrin with Pepsin—Fibrin was studied as an example
of a fibrous protein. In preliminary experiments, the protein was sus‑
pended in HCl and digestion carried out in a cellophane dialyzing sac as
described above. The results of two such experiments are given in Table
III, Experiments 1 and 2, in which the average number of amino acid
residues per peptide molecule varied over a range of 3.3 to 5.1 In some
other experiments, there was an even greater variation in the ratio of the
total amino nitrogen to the terminal amino nitrogen at different times

during digestion (in the range of 3 to 7), and this did not appear to be a function of the time of incubation. This variation may be due to the fact that the fibrin forms a gelatinous mass when suspended in HCl. More consistent results were obtained by preparing a homogenate of fibrin in HCl at pH 1.8, before the addition of the enzyme. The results of two such experiments are given in Table III, Experiments 3 and 4, and show a variation in the ratios from 3.7 to 4.4. The particular interest of these experiments on fibrin is the relatively high concentration of free amino acid

TABLE III

Digestion of Purified Bovine Fibrin by Crystalline Pepsin at pH 1.8

Experiment 1, 350 mg. of fibrin suspended in 20 cc. of HCl + 50 mg. of pepsin; Experiment 2, 250 mg. of fibrin suspended in 5 cc. of HCl + 50 mg. of pepsin; Experiment 3, 200 mg. of fibrin homogenized in 10 cc. of HCl + 10 mg. of pepsin; Experiment 4, 200 mg. of fibrin homogenized in 10 cc. of HCl + 10 mg. of pepsin.

Experiment No.	Time of sampling	Free amino acid nitrogen (COOH-N)	Terminal amino nitrogen (NH_2-N)	Per cent of NH_2-N as free amino acid	Total amino nitrogen (COOH-N)	$\dfrac{\text{COOH-N}}{NH_2\text{-N}}$, average No. of amino acid residues per peptide molecule (corrected for free amino acids)
	hrs.	*mg.*	*mg.*		*mg.*	
1	0.5	0.26	0.91	28	2.42	3.3
	1	0.28	2.48	11	7.7	3.3
	4	0.28	8.90	3	33.8	3.9
2	0.25	0.02	0.11	22	0.39	4.1
	0.75	0.10	0.74	13	2.50	3.7
	2	0.21	2.58	8	11.80	4.9
	3.5	0.24	4.20	6	20.5	5.1
3	0.3	0.07	0.38	17	1.31	4.0
	1	0.13	1.68	8	6.03	3.8
	3.5	0.24	3.09	8	11.05	3.8
4	0.5	0.07	0.69	11	2.38	3.7
	3	0.14	1.94	7	8.16	4.4

liberated, most of which occurs in the early stages of digestion. In the experiments reported in Table III, values from 11 to 28 per cent of the total amino nitrogen were present as free amino acid nitrogen after the first 15 to 20 minutes of digestion. After 1 hour, the rate of increase of free amino acid nitrogen was small as compared to that of the terminal peptide amino nitrogen. The possible release of non-protein amino nitrogen by homogenizing the fibrin was controlled by analyzing the dialysate of a sample of homogenate incubated without the addition of enzyme. No amino nitrogen was detected after 3.5 hours of incubation.

Removal of Digestion Products by Dialysis—The evidence that has been presented by Bergmann and his colleagues on enzymatic synthesis of

peptide bonds, and also on the effect of peptides acting as "cosubstrates," has frequently been advanced as an objection to the study of the peptides formed by enzymatic hydrolysis of proteins in relation to the protein structure or to the enzyme specificity.

One method of reducing the likelihood of these secondary reactions occurring is to remove the peptides by dialysis as they are formed during digestion. The rocker-perfusion method referred to in the experimental section was used for this purpose. The results of such an experiment, with serum albumin as substrate, are given in Table IV. The average value of

TABLE IV

Digestion of Bovine Serum Albumin by Pepsin in Which Peptides Are Removed by Dialysis

10 cc. of 5 per cent serum albumin + 10 mg. of pepsin.

Period of collection of dialysate	Terminal amino nitrogen (NH$_2$-N)	Total amino nitrogen (COOH-N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
hrs.	*mg.*	*mg.*	
0 −0.25	0.15	0.45	2.9
0.25–0.75	0.79	3.04	3.8
0.75–1.25	0.85	3.05	3.6
1.25–1.75	0.74		
1.75–2.25	0.60	2.28	3.8
2.25–3.25	1.12		
3.25–4.25	0.86	2.82	3.3
4.25–5.25	0.57	2.02	3.5
5.25–6.25	0.59		
6.25–7.25	0.44	1.95	4.4
7.25–8.25	0.37		
Average	... .		3.6

the number of amino acid residues per peptide molecule is not significantly different from the results found without dialysis, giving an average of 3.6 (Table II, Experiment 2). It is seen from these results that the amino nitrogen value in the first 15 minute dialysate is low (due to dilution by the HCl initially present in the dialyzing tube). In the following 3 hours, the total amino nitrogen found in the dialysate per unit time remained relatively constant.

One cannot ascertain from this experiment whether the rate of digestion or the rate of dialysis is the limiting factor. If the rate of dialysis is slow in relation to the rate of proteolysis, a high concentration of peptides will accumulate in the digestion vessel.

In Experiment 1, Table V, analyses were made at given times on the
peptides in the dialysate, Solution A, and also in the dialyzed solution,

TABLE V

Digestion of Bovine Serum Albumin by Crystalline Pepsin with and without Removal of Peptides by Dialysis

Experiment 1, 15 cc. of 3.3 per cent serum albumin + 10 mg. of pepsin; Solution A, dialysate; Solution B, dialyzed solution; Solution C, closed system of digestion. Experiment 2, 15 cc. of 3 3 per cent serum albumin + 2 mg of pepsin.

Experiment No.	Solution	Period of collection of dialysate and time of sampling	Terminal amino nitrogen (NH₂-N)	Total amino nitrogen (COOH N)	$\frac{\text{COOH-N}}{\text{NH}_2\text{-N}}$, average No. of amino acid residues per peptide molecule
		hrs.	*mg.*	*mg*	
1	A	0 –1	0 93	3 94	4 2
		1 –2	1.12	4 50	4 0
		2 –3	0 89	3 22	3 7
		3 –4	0 65	2 29	3 5
		4 –5	0 41	1 52	3 6
Average					3 8
	B	0 –0 5	7 46	24 8	3.3
		0 5 –1	7 30	22 8	3.0
		1 –2	5 67	20 8	3.7
		2 –3	4 08	11 9	3.0
		3 –4	3.82	16 5	4 3
		4 –5	2 50	10 7	4 3
Average					3 6
	C	0 –0.5	5 58	17 6	3 2
		0.5 –1	5.83	18 7	3 2
		1 –2	6 15	21 1	3 2
		2 –3	6 73	21 4	3 1
		3 –4	8 3	23 6	2 9
Average					3 1
2		0 –0 25	0 09	0 37	4 1
		0 25–0 5	0 35	1 34	3 8
		0 5 –1	0 56	1 86	3 3
		1 –2	1 17	3 38	2 9
Average					3 5

Solution B. Simultaneously, a digestion system was set up in a closed
vessel, Solution C (as used in all earlier experiments). 2 cc. aliquots of
Solutions B and C were withdrawn at the times indicated and neutralized

to stop digestion. These aliquots were then dialyzed against 10 cc. of water at 4° overnight. In this procedure, the use of a protein precipitant was omitted.

The results indicate that there is no significant difference in the peptide lengths obtained whether or not dialysis is employed. However, this experiment also shows that the rate of dialysis is inadequate for the complete removal of peptides as they are formed, since the dialyzed amino nitrogen values are much less than the corresponding values analyzed in the dialyzed digestion mixture. In order to decrease this accumulation of peptides, a similar system of dialysis as described above was used, but only 2 mg. of pepsin were added. The results are given in Table V, Experiment 2. The results show no significant difference from those reported in Experiment 1.

DISCUSSION

In the experiments reported here, we have not observed any significant variation in the size of the peptides formed during digestion. This is consistent with the "all or none" hypothesis advanced by Tiselius and Eriksson-Quensel discussed above. Although the evidence indicates that there is not a gradual degradation of the protein molecule, it cannot be overlooked that our analyses were performed on trichloroacetic acid or picric acid filtrates or the dialyzable fractions. Therefore, large molecules resulting from partial degradation could be present in the protein precipitate or in the non-dialyzable fraction.

The work of Petermann (10) suggests an alternate mechanism of proteolysis. From ultracentrifuge studies of the peptic digestion products of beef serum pseudoglobulin, at pH values from 2.7 to 4.5, she was able to demonstrate the presence of components of high molecular weight (probably halves and quarters of the protein molecule) under the more alkaline conditions. She suggests that at the optimum pH for enzymatic activity the breakdown may occur too rapidly to observe intermediate components. Bridgman (11), studying the peptic digestion of human γ-globulin by ultracentrifuge analysis, also identified half molecules of the protein. He obtained a maximum yield of the latter at pH 3.5.

The differences between these findings and our own and those of other workers may be due to the fact that proteolysis varies, depending on the structure of the protein substrate. It is of interest that fibrin (the only fibrous protein studied so far in relation to the mechanism of proteolysis) should behave differently from the other substrates in that it gives a greater release of free amino acids, and that this occurs in the initial stages of digestion.

In recent years, considerable work has been done on the partial acid

hydrolysis products of proteins in relation to protein structure. There has been very little effort, however, to apply enzymatic hydrolysis to such studies, although it is probable that one could get easily reproducible results by this method. The objections to the application of enzymatic hydrolysis in these studies has been discussed above, and it is felt that the use of some method for the continual removal of peptides from the digestion mixture as described in this paper may be of value in future development of work on these lines.

The work on the specificity of proteolytic enzymes by Bergmann and his colleagues has been applied to synthetic substrates. From these findings, the conception has arisen that the proteolytic enzymes have very restricted specificity. According to present experiments, however, as much as 30 per cent of the peptide bonds of serum albumin is hydrolyzed by pepsin. Therefore, a further application of the study of proteolysis may be made in relation to enzyme specificity by a study of the amino acid composition of some of the peptides formed.

SUMMARY

1. The digestion of crystalline bovine serum albumin and human γ-globulin by crystalline trypsin and of crystalline bovine serum albumin, purified fibrin, and twice recrystallized egg albumin by crystalline pepsin has been studied.

2. The average number of amino acid residues per peptide molecule formed during proteolysis was estimated. These values remained approximately constant throughout digestion. The significance of this finding in relation to the enzymatic mechanism is discussed.

3. The free amino acids liberated were also analyzed. Peptic digestion of fibrin showed a comparatively high concentration of free amino acids released in the first 30 minutes of digestion.

4. A system for the continual removal of the peptides from the digestion mixture by dialysis is described.

5. The possible applications of studies on the products of proteolysis of purified proteins are discussed.

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A REINVESTIGATION OF FLAVACIDIN, THE PENICILLIN PRODUCED BY ASPERGILLUS FLAVUS

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In a preliminary communication Fried, Koerber, and Wintersteiner (1) reported the results of their study in 1944 on the chemical nature of "flavacidin," the penicillin produced by *Aspergillus flavus*. The evidence then available seemed to indicate that flavacidin was a new penicillin not produced by *Penicillium notatum*; namely, 3-pentenylpenicillin, mixed with some benzylpenicillin (penicillin G).[1]

The tentative identification of the main component as 3-pentenylpenicillin was based on the following findings (3). Though the analyses of the crystalline sodium salt of flavacidin were not quite conclusive, they favored on the whole a composition $C_{14}H_{17-19}O_4N_2SNa$. The formula with 19 H atoms is that of the sodium salt of 2-pentenylpenicillin, R = $CH_3 \cdot CH_2 \cdot CH{=}CH \cdot CH_2^-$, one of the entities produced by *Penicillium notatum*, but a comparison of the x-ray diffraction pattern of a specimen of the latter compound, previously isolated at the Squibb Institute, with that of the flavacidin salt revealed such marked differences as to preclude identity with this penicillin. The flavacidin salt was then degraded to the penilloaldehyde, $R \cdot CO \cdot NH \cdot CH_2 \cdot CHO$ (I), by acid hydrolysis and subsequent treatment with mercuric chloride (4). The penilloaldehyde was isolated as the 2,4-dinitrophenylhydrazone. The analysis of this derivative conformed with the calculated values for the dinitrophenylhydrazone of a pentenylpenilloaldehyde (I, R = C_5H_9), but it strongly depressed the melting point of a penilloaldehyde dinitrophenylhydrazone which had been previously obtained in the same manner by degradation of crystalline sodium 2-pentenylpenicillinate from *Penicillium notatum* (5). Moreover, the x-ray diffraction patterns[2] of the two degradation products were un-

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[1] The nomenclature employed here conforms with that adopted in the forthcoming monograph, "The chemistry of penicillin," (2) and is based on the R group differentiating the various penicillins instead of on the arbitrary designation by letters or numbers hitherto used. In the new terminology, penicillin F is 2-pentenylpenicillin; dihydropenicillin F, *n*-amylpenicillin; penicillin G, benzylpenicillin; penicillin K, *n*-heptylpenicillin; and penicillin X, *p*-hydroxybenzylpenicillin.

[2] Measurements by N. C. Schieltz, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois; private communication, June 8, 1944.

mistakably different, and this was also the case when the dinitrophenylhy-
drazone of synthetic 3-hexenoylaminoacetaldehyde (I, R = $CH_3 \cdot CH_2 \cdot$
$CH = CH \cdot CH_2^-$) was substituted for that derived from 2-pentenylpenicil-
lin by degradation. On the other hand, the penilloaldehyde dinitro-
phenylhydrazone from flavacidin and the derivatives obtained from syn-
thetic 4-hexenoylaminoacetaldehyde (I, R = $CH_3 \cdot CH = CH \cdot CH_2 \cdot CH_2^-$)
showed close correspondence of the patterns, indicating that the precursor
of the former was 3-pentenylpenicillin. It was realized that this conclusion
would have to be substantiated by rigid characterization of the acid R·
COOH, but the amounts of crystalline salt obtained in 1944 did not suffice
for further degradation work.

The study was resumed in 1946 when a larger batch of fermentation
broth from *Aspergillus flavus* was prepared in the Squibb penicillin plant.
For the brown sugar-containing medium employed in the original work,
one containing lactose was substituted, a measure which raised the potency
of the broth from about 20 units to 70 units per cc. The culture filtrate was
worked up in the same manner as the 1944 batch; that is, by the usual
multiple solvent extraction procedure, except that some further fractiona-
tion was accomplished in the last step by conducting the extraction of the
final ether solution with sodium bicarbonate in three stages, corresponding
to a final pH of the resulting sodium salt solution of 6.0, 6.5, and 7.2, re-
spectively. Exploratory chromatographic and crystallization experiments
showed that only the first of these fractions, which represented 62 per cent
of the total units recovered as sodium salts, readily yielded crystalline
material by the isolation procedure used in 1944. However, it soon be-
came clear from analytical and ultraviolet absorption data that this crystal-
line product differed from that obtained in 1944 in that it contained pre-
ponderantly benzylpenicillin instead of a penicillin of the F type. After
purification by chromatographing on alumina, as in the isolation of benzyl-
penicillin (6) and of flavacidin (2), the benzylpenicillin was removed by
the triethylamine procedure (7). The material not precipitable by triethyl-
amine was converted into a crystalline ammonium salt, which was spectro-
graphically free from benzylpenicillin (absence of phenyl bands in the range
2500 to 2700 A). The analyses of this product, and those of a potassium
salt prepared from it, indicated that we were dealing with an amylpenicillin
rather than a pentenylpenicillin. The Craig distribution curve (8) of the
potassium salt (Fig. 1) showed but one maximum, but its shape, and the
trends in the calculated distribution coefficients (9) and in the *Bacillus
brevis-Staphylococcus aureus* assay ratios over the range of the curve de-
finitely indicated inhomogeneity. A sample shaken with hydrogen in the
presence of platinum oxide, conditions under which 2-pentenylpenicillin
is readily reduced to *n*-amylpenicillin (10), took up in sluggish reaction

about 20 per cent of the hydrogen volume required by an ammonium pentenylpenicillinate. On the assumption that the original product consisted mainly of the *n*-amylpenicillin mixed with a much lesser amount of a pentenylpenicillin, it was hydrolyzed with strong hydrochloric acid in order to secure and characterize the side chain fatty acids. The acidic fraction, which accounted for about 80 per cent of the ether-soluble products, consisted of substantially pure *n*-caproic acid as shown by analysis, physical properties, and conversion to the crystalline *p*-toluidide. There was no doubt, therefore, that the main component of the ammonium salt mixture was *n*-amylpenicillin and not a pentenylpenicillin. That the minor constituent revealed by counter-current distribution was a penicillin of the latter type could be surmised from the small but definite hydrogen uptake. The hexenoic acid derived from this entity was apparently converted, under the influence of the mineral acid used in the hydrolysis, into a lactone which remained in the neutral portion of the ether-soluble products, and was later lost by volatilization.
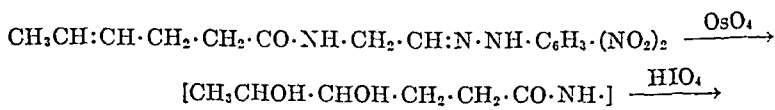
Next, the potassium salt, as well as the contents of Tubes 14 to 20 from the counter-current distribution experiment, which could be assumed to consist of substantially pure *n*-amylpenicillin, were degraded to the penilloaldehyde by the procedure mentioned earlier. The dinitrophenylhydrazones thus obtained were recrystallized till their melting points were constant (182.5° and 183.5°, respectively). As expected, neither preparation depressed the melting point (188°) of synthetic *n*-caproylaminoacetaldehyde dinitrophenylhydrazone. However, it was somewhat surprising to find that their mixtures with the penilloaldehyde dinitrophenylhydrazone obtained in 1944 (m.p. 182°) and with the sample of synthetic 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone used for comparison at that time likewise showed no melting point depression. A new specimen of the latter compound was then synthesized and carefully purified. Its melting point was somewhat higher (186°) than that of the old preparation, but again no depression was observed in mixture, either with the present penilloaldehyde dinitrophenylhydrazone or with the synthetic *n*-caproyl derivative. In the hope that the x-ray diffraction patterns would reflect the structural differences between the two synthetic compounds and thus achieve the desired differentiation, the two penilloaldehyde dinitrophenylhydrazone preparations from the present batch and the two synthetic compounds were submitted to Dr. G. L. Clark of the Department of Chemistry, University of Illinois, who kindly consented to have the necessary measurements carried out in his laboratory.[3] However, the diffraction patterns of the four preparations were practically identical, nor could

they be distinguished from the patterns obtained in 1944 in the Northern Regional Research Laboratory from the compounds then submitted (dinitrophenylhydrazones of the degradation product and of synthetic 4-hexenoylaminoacetaldehyde).

It was clear, then, that the dinitrophenylhydrazones of n-caproyl- and 4-hexenoylaminoacetaldehyde cannot be distinguished by either melting point or x-ray data, and that consequently the flavacidin obtained in 1944 could have been either 3-pentenylpenicillin or n-amylpenicillin. However, since there is no other conclusive evidence for the existence of a 3-pentenylpenicillin, whereas n-amylpenicillin is known to be a metabolic product of another aspergillic species, namely *Aspergillus giganteus* (11), as well as of *Penicillium notatum* (12), it appears highly probable that the entity isolated in 1944 was actually the latter penicillin. The fact that the analytical composition of the penilloaldehyde dinitrophenylhydrazone then obtained corresponded to the formula with 2 hydrogen atoms less was probably due to contamination with some of the phenylacetylaminoacetaldehyde derivative, originating in the small amount of benzylpenicillin in the flavacidin sodium salt.

The remainder of the study was concerned with adducing evidence for the nature of the R group in the minor component, presumably a pentenylpenicillin, present in the benzylpenicillin-free crystalline preparations. To attempt the isolation of this entity in form of a pure salt seemed impractical in view of the small proportion present and the difficulties attending the separation of such closely related penicillins. Similar difficulties could be anticipated in the separation of small amounts of the derived fatty acids; moreover, the danger of double bond shifts in hexenoic acids or of their conversion to lactones by the vigorous hydrolytic treatment necessary for their liberation could not be disregarded. We therefore resorted to an indirect but milder method, in which the penilloaldehyde dinitrophenylhydrazone mixture was used as the starting material, and which is exemplified below for 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone.

$$CH_3CH{:}CH \cdot CH_2 \cdot CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CH{:}N \cdot NH \cdot C_6H_3 \cdot (NO_2)_2 \xrightarrow{OsO_4}$$

$$[CH_3CHOH \cdot CHOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot NH \cdot] \xrightarrow{HIO_4}$$

$$CH_3 \cdot CHO \text{ (isolated as dinitrophenylhydrazone)}$$

To make sure that these reactions proceeded in the expected fashion, 4-hexenoic acid p-toluidide was treated with osmic acid in ether. The yield of pure 4,5-dihydroxycaproic acid p-toluidide was 46 per cent. Oxidation of the latter compound with periodic acid gave acetaldehyde, which was isolated as the 2,4-dinitrophenylhydrazone. The weight of the derivative corresponded to 65 per cent of the calculated amount. Similar experi-

ments, but without isolation and characterization of the intermediate glycols, were performed with 4-hexenoic acid itself and with 4-hexenoyl-aminoacetaldehyde dinitrophenylhydrazone. In the latter case the over-all yield of acetaldehyde was 48 per cent of the theoretical, showing that the side products which were undoubtedly found in both oxidation steps did not interfere with the isolation of the volatile aldehydic fragment. The choice of the penilloaldehyde dinitrophenylhydrazone as the starting product for the oxidative degradation was dictated by the necessity, on the one hand, of avoiding the complications to be expected from the presence of sulfur-containing products derived from the penicillamine moiety, and, on the other hand, of securing the moiety containing the R group in the form of a derivative which could be isolated in reasonably good yield, thus minimizing the danger of losing by fractionation a substantial part of the unsaturated component of the mixture.

When the procedure was applied to the total crude dinitrophenylhydrazone mixture obtained from benzylpenicillin-free crystalline material, only a small amount of volatile aldehyde was recovered as the dinitrophenylhydrazone after the second oxidation step. Lack of material prevented complete purification of the derivative, but its analytical properties left little doubt that the constituting aldehyde was propionaldehyde and not acetaldehyde, and hence the penicillin from which this fragment was derived was 2-pentenylpenicillin. This result renders it all the more probable that the 1944 flavacidin was n-amylpenicillin and not 3-pentenylpenicillin.

An incidental result was the isolation of 2-furoic acid from a chromatographic side fraction. It is interesting to note that this acid was also encountered by McKee and MacPhillamy (13) in their original work on the factor later called flavacidin. It has furthermore been found in impure penicillin from *Penicillium notatum* (14). Since 2-furoic acid does not seem to be a common metabolic product of molds, it may have its origin in the corn steep liquor used as a constituent of the medium in all these instances.

<div align="center">EXPERIMENTAL</div>

Microbiological Assays—Unless stated otherwise, the potency and unitage figures given in the following were obtained by plate assay with *Staphyloccus aureus* (Heatley) as the test organism and crystalline sodium benzylpenicillinate (Food and Drug Administration standard, 1667 units per mg.) as the standard. Differential assay denotes the ratio of the activity against *Bacillus subtilis* (rough strain) over that against *Staphylococcus aureus* as measured by plate assay (15). For some of the highly purified and crystalline preparations, the ratios of the minimal inhibiting concentrations for *Staphylococcus aureus* to *Bacillus brevis* to Organism E (16),

as measured by the broth dilution method of Donovick, Lapedes, and Pansy (17), are given. This ratio, designated St:B:E in the following, is 1:1.9:4.2 for benzylpenicillin, 1:3.8:6.9 for 2-pentenylpenicillin, and (on the basis of the results obtained in this work with about 75 per cent pure potassium *n*-amylpenicillinate) in the neighborhood of 1:3.5:12 for *n*-amylpenicillin.

Fermentation and Extraction—The fermentation of *Aspergillus flavus* was carried out in a large fermentation tank, with a medium which contained besides the usual inorganic constituents (KH_2PO_4 1.0 per cent, magnesium sulfate 0.55 per cent, chalk 1.0 per cent) 1.5 per cent of cheese whey as the lactose source, and 6.2 per cent of corn steep liquor. The broth was harvested 73 hours after inoculation, when its pH was 7.3 and its potency 70 units per cc. It was worked up by the usual multiple extraction procedure (amyl acetate $\rightarrow$ buffer $\rightarrow$ chloroform $\rightarrow$ buffer $\rightarrow$ ether). The final ether solution, which contained 74 per cent of the activity in the filtered broth, was fractionally extracted with sodium bicarbonate solution in such a manner that the final pH of the first extract was 6.0 (Fraction I); that of the second, 6.5 (Fraction II); and that of the third, 7.2 (Fraction III). The sodium salt obtained from the first extract represented 62 per cent of the units present in the ether and had a potency of 720 units per mg.; the differential assay ratio was 0.9, indicating the presence of a considerable amount of benzylpenicillin (differential assay = 1.0). For the reason pointed out above, only this fraction was thoroughly examined chemically.[4]

Chromatographic Purification—A typical experiment is described. 25 gm. portions of the crude sodium salt from the extract at pH 6.0 were dissolved in 150 cc. of acetone containing 5 per cent of water, and chromatographed on a column (6.5 × 47 cm.) of sulfuric acid-washed alumina (pH 4.5 in water suspension). The chromatogram was developed by washing with 3 liters of the same solvent. The distribution of pigments and of activity in a typical chromatogram was as follows: brown top zone (A), 7 cm.; light tan zone (B), 16 cm.; orange band (C), 1 cm.; colorless zone (D), 5 cm.; yellow band (E), 1 cm.; pale yellow zone (F), 17 cm. The

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[4] The corresponding data for the other two fractions are as follows: Fraction II, 23.5 per cent of activity, 745 units per mg., differential assay, 0.80; Fraction III, 10 per cent of activity, 366 units per mg., differential assay, 0.70. Preliminary chromatographic and crystallization experiments were carried out on both fractions by Dr. C. Glaser of the Squibb Manufacturing Laboratories. The crystalline product obtained from Fraction II in poor yield resembled similar material from Fraction I, particularly in regard to its high content of benzylpenicillin. Fraction III yielded small amounts of crystalline products containing relatively little benzylpenicillin; the analytical, Craig, and microbiological data indicated that they consisted of mixtures of penicillins of the F type with some benzylpenicillin and probably also *n*-heptylpenicillin.

column was cut into three sections, each of which was eluted with 0.2 M phosphate buffer of pH 7.0 at 5°. The eluted materials were converted into sodium salts in the usual way. The top section, SI, comprising Zone A, contained 17 per cent of the activity; the middle section, SII (Zones B, C, and D), 45 per cent; the bottom section, SIII (Zones E and F), 24 per cent.

Sodium salts obtained from Section SII, which represented the purest material and contained the bulk of the activity, readily yielded crystals on treatment with dry acetone. However, when their high content of benzylpenicillin became apparent, crystallization at this stage was omitted; instead, the buffer eluate from Section SII was chilled, acidified to pH 2 with phosphoric acid, and extracted with ether. This ether solution was then used directly for the fractionation with triethylamine.

Isolation of Benzylpenicillin—To a dry ether solution (160 cc.) of free penicillin (1.6 gm., representing Section SII from the chromatographic fractionation of 5 gm. of Fraction I) there was added in small portions a 10 per cent ethereal solution of triethylamine. The oily deposits initially formed were discarded. After the addition of an excess of the reagent the solution was allowed to stand at 4° for 12 hours. The crystalline triethylamine salt was collected (522 mg.) and recrystallized from hot chlorobenzene (18). It melted at 131–134° (decomposition) and showed the following microbiological characteristics: potency, 1310 units per mg.; differential assay ratio, 0.93; St:B:E, 1:1.7:5.2. The analysis of the desiccator-dry preparation was low in carbon, indicating contamination with an F type of penicillin. A larger amount of crude triethylamine salt (SII-A, 15.8 gm., from 60 gm. of Fraction I) was therefore converted into the sodium salt by transfer through ether. A portion of the lyophilized product (5.5 gm.) was treated with dry acetone, and the resulting semicrystalline mass (1.25 gm.) was recrystallized from aqueous *n*-butanol in the usual manner (19). Further recrystallization from aqueous acetone gave 0.9 gm. of pure sodium benzylpenicillinate assaying 1640 units per mg. (average of thirteen assays) and giving a St:B ratio (minimal inhibiting concentrations) of 1:1.55.

$C_{16}H_{17}O_4N_2SNa$. Calculated, C 53.92, H 4.81; found, C 54.15, H 4.99

Isolation and Properties of Crystalline Salts from Benzylpenicillin-Free Fraction—The ethereal filtrate from the triethylamine salt SII-A was chilled and extracted with an ice-cold solution of phosphoric acid till all the triethylamine was removed. The ether phase was washed with water and then extracted with several portions of dilute aqueous ammonium hydroxide till the pH of the combined extracts was 6.5. The lyophilized ammonium salt, which assayed 1470 units per mg. and represented 25 per cent of the

the presence of 5.6 per cent of a component with a high distribution coefficient, possibly n-heptylpenicillin. The more slowly moving component revealed by the deviation at the left side of the maximum and accounting for the remaining 20 per cent was probably for the most part 2-pentenylpenicillin.

Spectrophotometric Procedure for Detection and Estimation of Benzylpenicillin in Mixtures—The absence of significant amounts (>5 per cent) of benzylpenicillin in the ammonium salt SII-B1 was proved by means of a spectrophotometric procedure, which utilizes the fact that the phenyl bands originating in the benzyl group become much better defined when this penicillin is hydrolyzed with alkali to benzylpenicilloic acid. Whereas in the ultraviolet absorption curve of benzylpenicillin the phenyl bands at 252, 258, and 264 mµ appear superimposed on the slope of the end-absorption, which is still very intense in that region, alkaline hydrolysis causes recession towards lower wave-lengths of the high band responsible for the end-absorption, so that the phenyl bands become well resolved, and their relative intensities approach more nearly those seen in the spectrum of phenylacetic acid. The extinction coefficient of the main phenyl band at 258.5 mµ can then serve as a rough measure of the benzylpenicillin content of a mixture, while in the curve of the unhydrolyzed preparation it cannot be so evaluated. A correction must be applied for the contribution at this wave-length of the residual end-absorption band; *i.e.*, the extinction at 258 mµ given by an alkali-hydrolyzed sample of a pure penicillin possessing an aliphatic R group (preferably n-heptylpenicillin, which can be most easily freed from benzylpenicillin). Reasonably good quantitative results were thus obtained on mixtures containing various proportions of sodium benzylpenicillinate and ammonium n-heptylpenicillinate, the purity of which had been checked by the Craig counter-current distribution. With such mixtures, the greatest deviation of the determined from the actual benzylpenicillin content was ±5 per cent of the total penicillin present, and so the smallest content which could be detected with certainty was about 5 per cent. Three illustrative absorption curves are given in Fig. 2.

The experimental procedure was as follows: An accurately weighed sample (about 10 mg.) of the penicillin salt to be examined was dissolved in 5.0 cc. of aqueous 0.2 N sodium hydroxide. After standing at room temperature for 30 minutes, the solution was neutralized with 5.0 cc. of 0.2 N hydrochloric acid, and used as such or after suitable dilution with 0.2 N sodium chloride solution for the determination of the ultraviolet characteristics in the region 245 to 270 mµ. When little or no benzylpenicillin was expected to be present, the extinctions were measured over that range in 0.5 mµ intervals and the complete curve was plotted; so that small deviations

from the smooth, flat curve characteristic for benzylpenicillin-free preparations could be detected.

n-Caproic Acid from Ammonium Salt SII-B1—The ammonium salt (600 mg.) was hydrolyzed with boiling 20 per cent hydrochloric acid (25 cc.) for 18 hours, whereupon the solution was distilled with steam. The distillate was neutralized with sodium bicarbonate and extracted with ether. The neutral product recovered from the dried ether phase was a brown oil (43 mg.), most of which volatilized on standing for several days
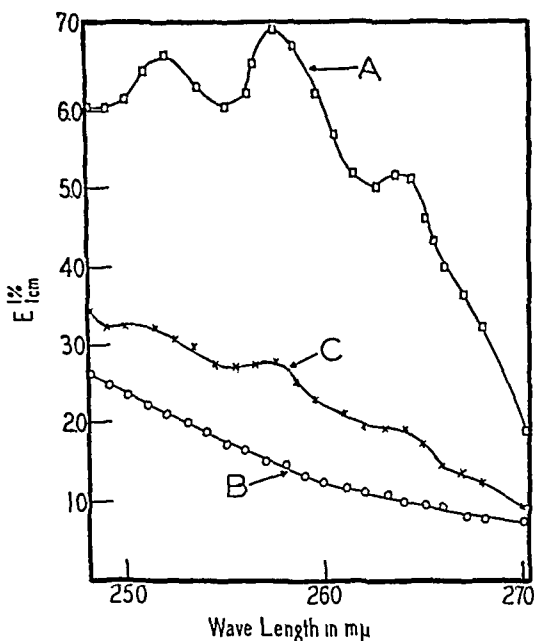

Fig. 2. Ultraviolet absorption curves of alkali-hydrolyzed penicillin. (1) Sodium benzylpenicillinate (Curve A); (2) ammonium *n*-heptylpenicillinate (Curve B); (3) mixture of (1) and (2), weight proportion 1:3 (Curve C).

in the evacuated desiccator. This product was apparently a lactone formed under the influence of the hot mineral acid from an unsaturated acid, presumably the 3-hexenoic acid.

The aqueous phase on acidification and extraction with ether yielded to the latter a liquid acid (152 mg.), which was purified by distillation *in vacuo* in a molecular still (b.p. 87° (bath temperature) at 9 mm.). When immersed in a freezing mixture, it crystallized and on thawing melted at −4° to −2°. The product did not react with alkaline permanganate or with bromine.

$C_6H_{12}O_4$. Calculated, C 62.04, H 10.41; found, C 62.02, H 10.31

The p-toluidide was prepared via the acid chloride, and after three recrystallizations from hexane melted at 74.5–75.5°. It did not depress the melting point of an authentic sample of n-caproic acid p-toluidide (75–76°).

$C_{13}H_{19}ON$. Calculated. C 76.08, H 9.33, N 6.83
Found. " 76.09, " 9.16, " 7.09

Penilloaldehyde 2,4-Dinitrophenylhydrazone from Potassium Salt SII-B2 —A solution of the salt (95 mg.) in 0.1 N sulfuric acid (15 cc.) was boiled under reflux for 2 hours, and after cooling mixed with a 5 per cent mercuric chloride solution (10 cc.). The resulting precipitate (penicillamine mercury mercaptide) was filtered off and washed with water. The combined filtrate and washings after demercurization with hydrogen sulfide, filtration, and aeration with nitrogen gas yielded with excess dinitrophenylhydrazine reagent (in ethanol-4 N HCl, 1:1) 44 mg. of a precipitate which after two recrystallizations from 75 per cent ethanol melted at 182–182.5° (decomposition).

The material recovered from Tubes 14 to 20 of the counter-current distribution experiment was combined and treated in the same manner. The purified dinitrophenylhydrazone thus obtained melted at 182.5–183.5° (decomposition).

n-Caproylaminoacetaldehyde 2,4-Dinitrophenylhydrazone—The diethyl-acetal of the adehyde was prepared from n-caproyl chloride and amino-acetaldehyde diethyl acetal in pyridine-ether according to Bentley *et al.* (20). The compound was purified by fractional distillation (b.p. 155–160° at 10 mm.) and then treated with a saturated aqueous solution of 2,4-dinitrophenylhydrazone in 5 N hydrochloric acid at 0°. The melting point of the once recrystallized dinitrophenylhydrazone was 187.5–188° (decomposition) and remained constant on further recrystallization.

4-Hexenoylaminoacetaldehyde 2,4-Dinitrophenylhydrazone—4-Hexenoic acid was prepared from "hydrosorbic acid" by the procedure of Letch and Linstead (21). The constants of the methyl ester (b.p. 55–55.6° at 19 mm.; n_D^{20} 1.4248) and of the acid (b.p. 107–108° at 16 mm.; n_D^{20} 1.4389) were in agreement with those given by these authors. The p-toluidide (22), prepared via the acid chloride, melted at 104°.

$C_{13}H_{17}ON$. Calculated. C 76.96, H 8.57, N 6.81
Found. " 76.81, " 8.43, " 6.89

A mixture of 4-hexenoic acid (1.97 gm.) and thionyl chloride (2.6 gm.) was allowed to stand at room temperature for 24 hours. After removal of excess reagent by aeration with nitrogen the residual oil was cooled to 0° and mixed with an ice-cold solution of aminoacetaldehyde diethyl acetal (1.84 gm.) in absolute ether (20 cc.). The solution was allowed to stand

at room temperature for 2 hours, whereupon it was filtered, washed with 20 per cent potassium carbonate solution and water, and dried over sodium sulfate. The residue obtained by removal of the solvent (4.0 gm.) was fractionally distilled *in vacuo*. The main fraction (b.p. 155–156° at 9 mm.) was treated with a saturated aqueous solution of 2,4-dinitrophenylhydrazine in 5 N hydrochloric acid at 0°. The resulting precipitate, after recrystallization from 60 per cent ethanol, melted at 185–186° (decomposition). The melting point did not change on further recrystallization.

$C_{14}H_{17}O_5N_5$. Calculated. C 50.15, H 5.11, N 20.9
                Found.   " 50.39, " 5.13, " 20.7

The melting points of the four dinitrophenylhydrazones prepared in this investigation, together with those of the penilloaldehydes from the 1944

TABLE I

2,4-Dinitrophenylhydrazone of	M.p.
	°C.
Penilloaldehyde from K salt SII-B2 (I)........................	182 –182.5
" " " " Tubes 14-20 (II)...........	182.5–183.5
" " 1944 flavacidin (III).....................	181.5–182*
" " 2-pentenylpenicillin (IV)................	178 –180
n-Caproylaminoacetaldehyde (V)............................	187.5–188
4-Hexenoylaminoacetaldehyde (VI)........................	185 –186

* Originally given as 180–180.5° (3).

flavacidin and from 2-pentenylpenicillin produced by *Penicillium notatum* (5), are listed in Table I. With the exception of IV, the preparations listed showed no melting point depression in mixture with one another. The melting points of the mixtures were generally those of the lower melting component, or slightly higher. Thus the mixtures of I and III and of I and V melted at 182–183°; that of I and VI, at 183–184°; that of III and V, at 181.5–183.5°; and that of the two synthetic compounds, V and VI, at 185–186°. In contrast, the penilloaldehyde dinitrophenylhydrazone from 2-pentenylpenicillin (IV) depressed the melting point of III to 174–175.5°, and that of the synthetic compound V to 172–172.5°.

Degradation of 4-Hexenoic Acid and Derivatives to Acetaldehyde. (a) *With 4-Hexenoic Acid*—To a solution of 4-hexenoic acid (445 mg.) in absolute ether (12 cc.) there was added osmium tetroxide (1.0 gm.) in 5 cc. of the same solvent. A brown precipitate formed immediately. The mixture was allowed to stand at room temperature for 2 days in the dark. The residue obtained by removal of the solvent *in vacuo* was decomposed by boiling with a solution of sodium sulfite (15 gm.) in 200 cc. of 50 per

cent aqueous ethanol for 4 hours. The resulting precipitate was removed by filtration and leached with four 50 cc. portions of ethanol. The residue obtained by evaporation of the combined filtrate and alcoholic solutions was freed from inorganic salts by repeated extraction with absolute ethanol. The extracts were brought to dryness *in vacuo*, yielding a yellow oil which was used without further purification for the reaction with periodic acid.

The consumption of periodate was determined by the method of Rappaport, Reifer, and Weinmann (23); 17.7 mg. consumed in fast reaction 26.6 mg. of KIO_4 (24 hour value); required for 4,5-dihydroxyhexanoic acid, 27.7 mg.

The glycol (220 mg.) was treated with periodic acid (620 mg.) in aqueous solution (12.5 cc.). The volatile reaction products were entrained in a current of nitrogen which passed through a 0.4 per cent solution of 2,4-dinitrophenylhydrazine in ethanol-4 N hydrochloric acid, 1:1, till no further precipitate was formed. The crude hydrazone (collected after 24 hours aeration, 244 mg., 73.7 per cent of the calculated amount) melted at 136° decomposition) after drying at room temperature. On recrystallization from aqueous ethanol, it yielded three successive crops of crystals differing in their melting points (147–151°; 142–143°; 145–149°). These were combined and chromatographed in chloroform solution on alumina. The bulk of the material was recovered from the bottom of the column by elution with ethanol as two fractions melting at 138–140° and 141–142°, respectively. Rechromatographing the combined fractions in benzene-hexane solution indicated that it was essentially homogeneous, though it now melted at 145–150°. It appears that this preparation consisted preponderantly of the low melting modification of acetaldehyde 2,4-dinitrophenylhydrazone (24), for on drying at 100° *in vacuo* the melting point rose steadily till it became constant at 154–156°.

$C_8H_8O_4N_4$.   Calculated.   C 42.86, H 3.60, N 25.0
                  Found.   " 42.68, " 3.51, " 24.8

(*b*) *With 4-Hexenoic Acid p-Toluidide*—A solution of the toluidide (1.444 gm.) in absolute ether (120 cc.) containing osmium tetroxide (2 gm.) was allowed to stand in the dark for 4 days, whereupon it was worked up as described under (*a*), but with double the amounts of sodium sulfite and solvents. The final product was a brown syrup which was decolorized with charcoal in ethanol and then crystallized from hot water. The *4,5-dihydroxycaproic acid p-toluidide* thus obtained melted at 150–150.5° (decomposition). Recrystallization did not raise the melting point. The yield of pure product was 780 mg. (46 per cent).

$C_{13}H_{19}O_3N$.   Calculated.   C 65.80, H 8.07, N 5.94
                      Found.   " 65.77, " 8.31, " 5.77

The toluidide (221 mg.) was dissolved in ethanol (2 cc.) and a solution of periodic acid (314 mg.) in water (4 cc.) was added. Aeration with nitrogen after 5 hours standing into dinitrophenylhydrazine reagent yielded 114 mg. of the acetaldehyde derivative, m.p. 147–150°, and an additional 28 mg. after 24 hours, the total yield corresponding to 65 per cent of the calculated amount.

(c) *With 4-Hexenoylaminoacetaldehyde 2,4-Dinitrophenylhydrazone*—To a solution of the hydrazone (160 mg.) in 7 cc. of dry pyridine (25) osmium tetroxide (250 mg.) dissolved in absolute ether (4 cc.) was added. After 2 days the mixture was worked up as described under (a). The crude reaction product (120 mg.) was used without further purification for the reaction with periodic acid (151 mg.). The solvent used was 23.5 per cent aqueous ethanol (8.5 cc.). After 6.5 hours 31 mg. and after 24 hours an additional 20 mg. of acetaldehyde dinitrophenylhydrazone, m.p. 143–144°, were collected. Recrystallization from aqueous ethanol (m.p. 149–150°) followed by heating at 100° *in vacuo* raised the melting point to 153–157°. The identity of the product was confirmed by elementary analysis.

A blank experiment carried out with the above amounts of periodic acid and 23.5 per cent ethanol yielded 17.5 mg. of a brown precipitate which possibly contained some acetaldehyde dinitrophenylhydrazone derived from the ethanol by an abnormal type of oxidation. In a similar blank run with aqueous dioxane no such precipitate was formed. This solvent was therefore used in the periodic acid step of the degradation experiment described below.

Oxidative Degradation of Penilloaldehyde Dinitrophenylhydrazone Mixture from Benzylpenicillin-Free Fraction—The starting product was a crystalline, benzylpenicillin-free ammonium salt which was prepared from Fraction I in the same manner as the ammonium salt SII-B1. It was degraded to the penilloaldehyde as described for the potassium salt SII-B2, except that the demercurization with hydrogen sulfide prior to the addition of the dinitrophenylhydrazine reagent was omitted. The crude dinitrophenylhydrazone (1.3 gm., m.p. 170–173°, decomposition) was treated with osmium tetroxide in pyridine-ether as in the procedure described in section (c). The reaction product, isolated in the usual manner, was a yellow oil weighing 750 mg. It was dissolved in pure dioxane (20 cc.), and after the addition of an aqueous solution (40 cc.) of periodic acid (1.4 gm.) a stream of nitrogen was passed through the clear solution into a receiver containing a 0.4 per cent solution of dinitrophenylhydrazine in ethanol-2 N hydrochloric acid, 1:1. The orange-colored crystals collected after 24 hours of aeration weighed 16 mg. and melted at 133–134° (decomposition, hot stage). They were recrystallized first from absolute ethanol and then from glacial acetic acid (m.p. 142–144°, hot stage). Lack of material prevented further

purification. A mixture with an authentic specimen of propionaldehyde 2,4-dinitrophenylhydrazone (m.p. 148–151°) melted at 143–145° (decomposition, hot stage).

$C_9H_{10}O_4N_4$. Calculated. C 45.38, H 4.23, N 23.5
$C_8H_8O_4N_4$.   "   " 42.86, " 3.60, " 25.0
          Found.   " 44.40, " 3.92, " 23.6

On a molar basis, the yield of propionaldehyde from the dinitrophenylhydrazone mixture was 1.85 per cent, which corresponds to only a small fraction of the assumed pentenylpenicillin content of the comparable ammonium salt SII-B1 (about 20 per cent). This discrepancy is undoubtedly in part due to losses sustained in the three steps of the degradation procedure; the yield of penilloaldehyde dinitrophenylhydrazone was only 60 per cent of the amount calculated for ammonium n-amylpenicillin, and, to judge from the experiment with 4-hexenoylaminoacetaldehyde dinitrophenylhydrazone, in which the yield of the acetaldehyde derivative was 47.5 per cent, even greater losses probably occurred in the following two oxidative steps. Moreover, some fractionation, entailing a comparatively greater loss of the unsaturated component, may have taken place in the preparation of the penilloaldehyde hydrazone mixture. However, in view of the complex procedure employed in the preparation of the benzylpenicillin-free salt from Fraction I, it is also quite possible that the pentenylpenicillin content of the ammonium salt used for the above experiment was markedly lower than that of SII-B1, and that this was the chief reason for the unexpectedly low over-all yield of propionaldehyde.

Isolation of 2-Furoic Acid—In a chromatographic experiment starting from 30 gm. of Fraction I, the dark brown band forming the lower part of Zone A was eluted separately with phosphate buffer. The eluate was subjected to the same procedure, inclusive of the precipitation with triethylamine, as the middle section of the chromatogram (Section SII). The lyophilized ammonium salt (1.2 gm., 435 units per mg.) obtained from the triethylamine filtrate on treatment with absolute acetone yielded crystalline material which was recrystallized from aqueous acetone in the usual manner. The resulting crystalline salt (408 mg., m.p. 188–189°, decomposition) was biologically inactive, and was identified as ammonium 2-furoate by absorption spectrum (ϵ_{max}. 11,000 at 245 mμ, in water), analysis, and conversion to the free acid.

$C_5H_7O_3N$. Calculated. C 46.51, H 5.46, N 10.85
          Found.   " 46.72, " 5.35, " 10.54

The acid melted at 128–129° and did not depress the melting point of an authentic sample of 2-furoic acid.

$C_5H_4O_3$. Calculated, C 53.58, H 3.60; found, C 53.89, H 3.81

DISCUSSION

The results of this investigation show that *Aspergillus flavus* under suitable culture conditions equals *Penicillium notatum* in its capacity to elaborate various penicillin species simultaneously. Two of these, benzylpenicillin and *n*-amylpenicillin, were conclusively identified, and satisfactory evidence for the presence of a third, 2-pentenylpenicillin, was adduced. It is noteworthy that *n*-amylpenicillin was first encountered in nature as the metabolic product of another *Aspergillus* species, *Aspergillus giganteus*. The partially purified penicillin-like antibiotic obtained from this source by Philpot (11) in 1943 was originally termed gigantic acid; it was subsequently shown to be *n*-amylpenicillin by conversion to the penillic acid and crystallographic identification of the latter with "dihydro-*n*-penillic acid" (10). Much more recently Salivar, Bogert, and Brown (12) have demonstrated the occurrence of *n*-amylpenicillin in submerged culture filtrates of *Penicillium notatum* (strain Q-176) by isolation and degradation to *n*-caproic acid.

Though it is difficult to estimate the proportion in which the three identified penicillins were present, there is no doubt that benzylpenicillin preponderated, not only in the crude sodium salt fraction examined but also in the total mixture extracted from the broth. In contrast, the crystalline flavacidin sodium salt isolated in 1944 contained only small amounts of benzylpenicillin, although the chromatographic and crystallization procedures used had proved to be highly effective in the earlier isolation of this entity from *Penicillium notatum* broth. The greater abundance of this species in the present batch is probably to be ascribed to the use of lactose instead of brown sugar in the culture medium, a change which may have resulted in better utilization of the benzylpenicillin precursors supplied with the corn steep liquor. *Aspergillus flavus* is not the only representative of the genus *Aspergillus* capable of producing this penicillin. Arnstein and Cook (26) have recently identified "parasiticin," the antibiotic produced by *Aspergillus parasiticus* in surface culture on a medium containing glucose and corn steep liquor, as benzylpenicillin.

Salivar, Bogert, and Brown (12) in their detailed study of crystalline ammonium salt mixtures from *Penicillium notatum* (strain Q-176) demonstrated the presence in their material of a penicillin closely allied with 2-pentenylpenicillin, which on the basis of our original work on flavacidin they assumed to be 3-pentenylpenicillin. This product showed a somewhat higher distribution coefficient in the Craig ether-buffer system than did 2-pentenylpenicillin, but, like the latter, yielded on acid hydrolysis the lactone of 4-hydroxycaproic acid. Since the lactone could arise from either 3- or 4-hexenoic acid, it appeared possible that 3-pentenylpenicillin was involved. However, since the fraction in question may have contained

some 2-pentenylpenicillin, and the yield of lactone relative to that obtained from the fraction definitely identified as 2-pentenylpenicillin was not stated, the evidence on this point is far from conclusive. Nevertheless, it is quite possible that future work may yet definitely establish the occur-rence of 3-pentenylpenicillin in the penicillin mixtures produced by *Penicillium notatum*, or for that matter in such derived from *Aspergillus flavus*. In any case, the distinctive term "flavacidin," which was retained merely for convenience of reference pending conclusive identification of the entity in question, should now be abandoned.

SUMMARY

Aspergillus flavus grown in submerged culture on a medium containing lactose and supplemented with corn steep liquor produced a mixture of penicillins, in which benzylpenicillin greatly preponderated. The crystal-line fractions obtained after removal of the benzylpenicillin were shown to consist for the most part of *n*-amylpenicillin. Evidence has been adduced by oxidative degradation for the presence in these fractions of small amounts of 2-pentenylpenicillin.

The 2,4-dinitrophenylhydrazones of *n*-caproylaminoacetaldehyde and of 4-hexenoylaminoacetaldehyde do not mutually depress their melting points, and give practically identical x-ray diffraction patterns. In view of this finding it appears probable that the "flavacidin" which was isolated in 1944 (1, 3) was *n*-amylpenicillin and not, as was then assumed, 3-pen-tenylpenicillin.

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BENZYLPENICILLENIC ACID AS AN INTERMEDIATE IN THE SYNTHESIS OF BENZYLPENICILLIN (PENICILLIN G)*

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During the war several English and American laboratories participated in a study of the structure and synthesis of penicillin (1, 2). In this work it was noted that when the methyl ester of benzylpenicillin (I) (see Fig. 1) was treated with mercuric chloride in ether solution and the resulting mercury derivative (II) was decomposed with hydrogen sulfide, a neutral, amorphous product was obtained. This crude product possessed an absorption peak in the ultraviolet at 320 mμ ($E_M = 13,700$)[1] and was degraded by sodium hydroxide to the sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone. For these and other reasons the product was assigned structure (III) and was given the trivial name methyl D-benzylpenicillenate (3).

After the close of the war, studies were continued in this Laboratory on the synthesis and the mechanism of synthesis of benzylpenicillin from D-penicillamine hydrochloride (V) and 2-benzyl-4-methoxymethylene-5(4)-oxazolone (IV) (4, 5). It was found that when the two compounds were allowed to react in pyridine containing triethylamine, a biologically inactive, amorphous product was obtained. However, when this product was heated in pyridine containing pyridinium chloride, benzylpenicillin was formed in small yield. The intermediate product possessed an ultraviolet absorption spectrum similar to that described for natural methyl D-benzylpenicillenate (III).[2] So far attempts to isolate the intermediate compound in crystalline form have not been successful. During the course of fractionation studies on this product, the formation of D-benzylpenillic acid (VI) was encountered (6). This D-benzylpenillic acid was identical with that formed by rearrangement of benzylpenicillin.

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[1] Throughout this paper E_M is the molar absorption coefficient and is equal to D/cd, where D is log I_0/I, c is concentration in moles per liter, and d is cell thickness in cm.

[2] In order to simplify phraseology in this article, D-benzylpenicillenic acid which has been prepared by rearrangement of benzylpenicillin is called "natural" D-benzylpenicillenic acid in contrast to that which has been prepared by total synthesis.

When DL-penicillamine hydrochloride was used instead of D-penicillamine hydrochloride in the condensation with the oxazolone (IV) in pyridine containing triethylamine, it was possible to isolate a crystalline compound from the reaction mixture. Since this compound had an absorption peak at 322.5 mμ (E_M = 26,000 to 28,000) and other properties in agreement with those of natural methyl D-benzylpenicillenate, it was called DL-benzylpenicillenic acid (IIIa) (7).

The crystalline DL-benzylpenicillenic acid (IIIa) rearranged in alcoholic solution to give a racemic benzylpenillic acid (VI). When the DL-benzylpenicillenic acid was heated in pyridine containing pyridinium chloride, antibiotic activity was produced, and furthermore in an amount proportional to the amount of D-benzylpenicillenic acid present. This ability to produce antibiotic activity was retained unchanged through repeated recrystallizations of the DL-benzylpenicillenic acid (7).

Since these results indicated that benzylpenicillenic acid (IIIa) was an intermediate in the synthesis of penicillin, it was desirable to demonstrate that the synthetic D-benzylpenicillenic acid was identical with natural D-benzylpenicillenic acid formed by rearrangement of benzylpenicillin.

Previous attempts in this and other Laboratories to obtain either natural or synthetic D-benzylpenicillenic acid in crystalline form had been unsuccessful (3, 5). Thus we were faced with the problem of trying to establish identity between two amorphous compounds. Since DL-benzylpenicillenic acid had been obtained in crystalline form, it occurred to us that amorphous D-benzylpenicillenic acid, either natural or synthetic, might be converted to a crystalline product by mixing it with an equivalent amount of amorphous L-benzylpenicillenic acid. Thus one might expect to obtain, on the one hand, a crystalline DL-benzylpenicillenic acid in which the D moiety arose by rearrangement of benzylpenicillin and, on the other hand, a crystalline DL-benzylpenicillenic acid in which the D moiety arose by synthesis from D-penicillamine (V) and the oxazolone (IV). In both cases, the L moiety would consist of synthetic L-benzylpenicillenic acid. If these two crystalline DL-benzylpenicillenic acids could be shown to be identical, then it would necessarily follow that the natural D-benzylpenicillenic acid was identical with the synthetic D-benzylpenicillenic acid.

Methyl benzylpenicillenate (III) had been prepared by the action of mercuric chloride on the methyl ester of benzylpenicillin (3). Since saponification of a compound as unstable as methyl benzylpenicillenate did not seem feasible, it was decided to investigate the action of mercuric chloride on sodium benzylpenicillin. If the reaction with sodium benzylpenicillin proceeded in a fashion analogous to that with the ester, one would expect to obtain D-benzylpenicillenic acid directly. Investigators at the Abbott Laboratories (3) had reported that treatment of sodium benzyl-

penicillin in aqueous solution with mercuric chloride resulted in the precipitation of a mercury derivative. This product possessed an absorption peak in dioxane at 320 mμ (E_M = 15,400) (3). The material was probably the mercury derivative (IIa) of D-benzylpenicillenic acid, and therefore a study of this reaction was initiated. Conditions were developed by which a mercury derivative (IIa) with an absorption peak in dioxane at 320 mμ (E_M = 18,000 to 19,000) and in ethanol at 342 mμ (E_M = 21,000 to 23,000) could be obtained from benzylpenicillin. This mercury derivative (IIa) was successfully converted to crude D-benzylpenicillenic acid (IIIa) by treating a suspension of the compound in a water-ethyl acetate mixture with hydrogen sulfide. The amorphous D-benzylpenicillenic acid which was isolated from the ethyl acetate layer possessed an absorption peak at 322.5 mμ (E_M = 17,600). When this crude D-benzylpenicillenic acid was allowed to stand in methanol, it rearranged to D-benzylpenillic acid (VI). In this respect it behaved similarly to the D-benzylpenicillenic acid prepared by synthesis from D-penicillamine (V) and the oxazolone (IV) (6).

Preliminary experiments were undertaken to determine whether crystalline DL-benzylpenicillenic acid could be obtained from a mixture of amorphous, synthetic D- and L-benzylpenicillenic acids. At first some difficulty was encountered in obtaining crystalline material upon admixture of the D and L compounds in solution. Presumably this was due to impurities present in these preparations. This difficulty was eliminated to a large degree when a method of partial purification of the crude penicillenic acids, based upon solvent extraction, was devised. It was noted that when a chloroform solution of the crude benzylpenicillenic acid was shaken with an equal volume of 2 M phosphate buffer solution at pH 5.4, a portion of the impurity went into the buffer layer. Although some of the benzylpenicillenic acid was either extracted or destroyed by this procedure, the benzylpenicillenic acid remaining in the chloroform layer was considerably purer than the starting material. The use of this information made it possible to isolate crystalline DL-benzylpenicillenic acid from a mixture of the synthetic D and L compounds.

Natural D-benzylpenicillenic acid was mixed with an equivalent amount of synthetic L-benzylpenicillenic acid. After a chloroform solution of this mixture had been purified by the extraction procedure, DL-benzylpenicillenic acid was isolated from the solution in crystalline form. This DL-benzylpenicillenic acid was identical in melting point, mixed melting point, and in infra-red and ultraviolet absorption spectra with the DL-benzylpenicillenic acid synthesized from DL-penicillamine (7) and also with material prepared from a mixture of synthetic D- and L-benzylpenicillenic acids. In addition, the DL-benzylpenicillenic acid (IIIa), in which the D moiety was natural, rearranged in methanolic solution to racemic benzylpenillic acid (VI).

When the DL-benzylpenicillenic acid containing natural D-benzylpenicillenic acid was heated in pyridine and pyridinium chloride, antibiotic activity was produced. The amount of antibiotic activity was equal, within experimental error, to that produced under similar conditions from synthetic DL-benzylpenicillenic acid. Synthetic L-benzylpenicillenic acid did not give rise to antibiotic activity under these conditions. Therefore, in the case of the DL-benzylpenicillenic acid, the activity must have arisen entirely from the D moiety of the compound.

These results prove that synthetic D-benzylpenicillenic acid is identical in all respects with natural D-benzylpenicillenic acid. Moreover the production of antibiotic activity, previously shown (2) to be due to benzylpenicillin, in identical amounts from two samples of crystalline DL-benzylpenicillenic acid in which the D moiety was prepared in two altogether different ways indicates beyond a reasonable doubt that *benzylpenicillenic acid, and not a small impurity present in the preparation, is an intermediate in the synthesis of benzylpenicillin* from penicillamine (V) and the oxazolone (IV). In any event, these data, in connection with those of other experiments cited above (2, 4, 5), demonstrate that benzylpenicillin may be rearranged to an antibiotically inactive product which under certain conditions can be converted in small part back to benzylpenicillin. The relationships discussed here are illustrated on the basis of the β-lactam formula for penicillin in Fig. 1.

The present communication also contains evidence as to the nature of the racemic benzylpenillic acid obtained by rearrangement of DL-benzylpenicillenic acid (7). Since there are three asymmetric carbon atoms in benzylpenillic acid, there are four possible racemic forms of this compound. Admixture of equal quantities of synthetic D- and L-benzylpenillic acids gave rise to crystalline DL-benzylpenillic acid that was identical with the material prepared by rearrangement of DL-benzylpenicillenic acid. These results demonstrate that the racemic benzylpenillic acid formed by rearrangement of DL-benzylpenicillenic acid contains a D moiety which is identical with the D-benzylpenillic acid produced by rearrangement of D-benzylpenicillin.

It should be pointed out that, since benzylpenicillenic acid (IIIa) can rearrange to benzylpenillic acid (VI), it is possible that the rearrangement of D-benzylpenicillin (Ia) to D-benzylpenillic acid (VI) in aqueous solution at pH 2 (8) takes place through the intermediate formation of D-benzylpenicillenic acid (IIIa).

<div align="center">

EXPERIMENTAL[3]

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D- *and* L-*Benzylpenicillenic Acids*—To a mixture of 3.04 gm. (0.015 mole) of L-penicillamine hydrochloride hydrate and 3.04 gm. (0.014 mole) of

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[3] All melting points are corrected capillary melting points.

2-benzyl-4-methoxymethylene-5(4)-oxazolone were added 225 cc. of pyridine, and solution was effected by swirling the mixture. After addition of 24 cc. of triethylamine, the mixture was heated at 65–70° for 20 minutes.

The yellow solution was distilled *in vacuo* in a stream of nitrogen at a bath temperature of 50° until the solvents were removed. A solution of the residue in 300 cc. of chloroform was shaken with 150 cc. of 2 M phosphate buffer solution at pH 1.6 (prepared by admixture of equal volumes of
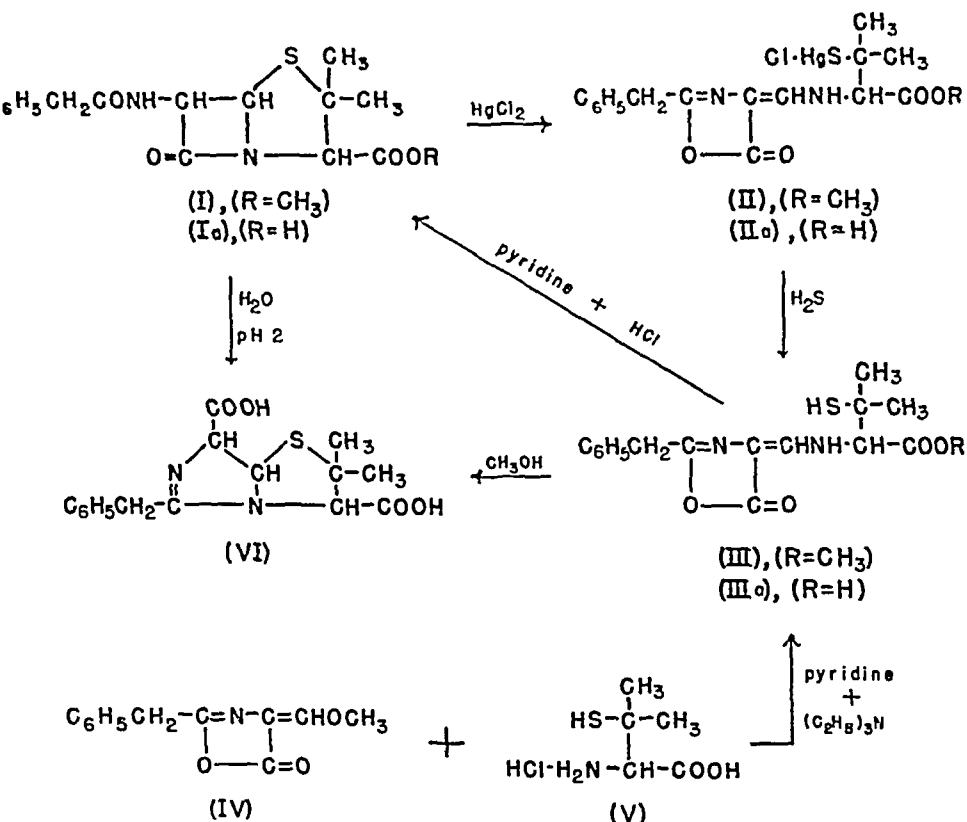

FIG. 1. Some reactions of benzylpenicillenic acid

2 M H_3PO_4 and 2 M NaH_2PO_4). Then the chloroform layer was shaken for 1 minute with two 150 cc. portions of 2 M phosphate buffer solution at pH 5.4.[4] The separated chloroform layer was dried over anhydrous $MgSO_4$ for 20 minutes, filtered by suction, and distilled *in vacuo* almost to dryness. The residual gum was dissolved in 30 cc. of chloroform and added gradually

[4] This buffer solution was prepared by admixture of 81 volumes of 2 ᴘО₄ with 19 volumes of 2 M K_2HPO_4. The resulting solution gave a pH   ᴜen measured without dilution with the glass electrode.

to 600 cc. of agitated hexane. The precipitate which formed was immediately filtered and dried *in vacuo* at room temperature. The weight of amorphous L-benzylpenicillenic acid ranged from 3.10 to 3.65 gm. (66 to 78 per cent); $E_M = 15,900$ to $17,000$ at 320 mμ in 95 per cent ethanol.[1] The maximum in the ultraviolet absorption peak occurred at 322.5 mμ. The specific rotation of the product varied slightly with different preparations, having an average value of about $[\alpha]_D^{22} = -82°$ (1.3 per cent solution in 95 per cent ethanol). Since the rotation of ethanolic solutions of either of the enantiomorphs of benzylpenicillenic acid gradually increased in value with time, the rotations were determined as soon as possible (within 15 minutes) after the solutions had been prepared.

D-Benzylpenicillenic acid was prepared from D-penicillamine hydrochloride hydrate in the manner described for the L acid. The specific rotation was approximately equal in amount but opposite in sign to that found for L-benzylpenicillenic acid.

Purification of DL-*Benzylpenicillenic Acid by Extraction*—Synthetic D- and L-benzylpenicillenic acids having approximately the same absorption at 320 mμ ($E_M = 16,500$) were selected for this experiment. A mixture of 100 mg. of each compound was dissolved in 50 cc. of distilled chloroform. The chloroform solution was shaken for 1 minute with 50 cc. of 2 M phosphate buffer solution at pH 5.4.[4] Aliquots for determination of ultraviolet absorption were removed from the chloroform solution before and after the extraction. The results of the absorption measurements indicated that 21 per cent of the benzylpenicillenic acid was removed or destroyed by the extraction. After the chloroform layer had been dried over anhydrous $MgSO_4$, it was filtered and concentrated to dryness *in vacuo*. The residue weighed 98 mg. (49 per cent) and possessed a molar absorption at 320 mμ of $E_M = 20,100$. Thus the extraction removed from the chloroform 51 per cent of the material on a weight basis but only 21 per cent of the benzylpenicillenic acid on an absorption basis. An additional extraction with the pH 5.4 buffer solution did not effect appreciable further purification.

Preparation of DL-*Benzylpenicillenic Acid by Admixture of Synthetic Enantiomorphs*—To 300 cc. of ice-cold chloroform were added 0.73 gm. of amorphous L-benzylpenicillenic acid ($E_M = 15,900$ at 320 mμ, $[\alpha]_D^{22} = -82°$) and 0.70 gm. of amorphous D-benzylpenicillenic acid ($E_M = 16,600$ at 320 mμ; $[\alpha]_D^{22} = +80°$). These quantities were equivalent on the basis of molar absorption coefficients. The solution was shaken for 1 minute with 100 cc. of ice-cold 10 per cent H_3PO_4, and then was shaken with 300 cc. of ice-cold 2 M phosphate buffer solution at pH 5.4.[4] After 10 minutes (to allow complete separation of phases) the lower layer was drawn off, placed in an ice bath, and dried with anhydrous $MgSO_4$ for 20 minutes. The desiccant was then filtered by suction, and the clear, yellow solution was

evaporated *in vacuo* in the absence of air of ebullition in a bath at 40–60°. Before half of the solvent was evaporated, the solution became cloudy and crystallization commenced. The mixture was concentrated to a volume of about 10 cc., allowed to stand in an ice bath for 40 minutes, and filtered. The white crystals were washed three times with a total of 5 cc. of distilled chloroform and dried *in vacuo* at room temperature; weight, 328 mg. (23 per cent); m.p. 131–133° (with decomposition); $E_M = 24,600$ at 320 mμ in 95 per cent ethanol.

When 298 mg. of this material were warmed for 5 minutes in 15 cc. of dry ethyl acetate and finally boiled for a minute, all but a trace of substance dissolved. The solution was filtered with the aid of gentle suction into a tared centrifuge tube and left in the cold. After 18 hours the white crystals were collected by centrifugation and dried *in vacuo;* weight, 178 mg. (60 per cent); m.p. 136–137° (with decomposition); $E_M = 26,600$ at 320 mμ in 95 per cent ethanol; absorption peak located at 322.5 mμ; $[\alpha]_D^{22} = 0°$ (0.35 per cent solution in 95 per cent ethanol). The melting point of this

TABLE I

Reaction of Mercuric Chloride with Sodium Benzylpenicillin

Concentration of reactants	Yield	E_M at 320 mμ
mole per l.	*per cent*	
0.0833	92	14,500
0.0417	89	16,000
0.0208	91	17,600
0.0104	85	18,500

DL-benzylpenicillenic acid was not depressed upon admixture with DL-benzylpenicillenic acid prepared from DL-penicillamine (7).

Effect of Concentration on Reaction of Mercuric Chloride with Sodium Benzylpenicillin—Samples of sodium benzylpenicillin (0.25 mM) were dissolved in various amounts of water. An aqueous solution of mercuric chloride containing 0.25 mM was added to each penicillin solution and the final volume was noted. The solutions were allowed to stand at room temperature (25°) for 2 hours and then refrigerated at 5° for 16 hours. The precipitates of the mercury derivative (IIa) were collected by filtration and dried *in vacuo* over phosphoric anhydride at room temperature. The yield and molar absorption coefficient of each material at 320 mμ in dioxane are shown in Table I.

Preparation of Mercury Derivative (IIa) from Sodium Benzylpenicillin—To 1.78 gm. (0.005 mole) of sodium benzylpenicillin dissolved in 100 cc. of water were added 1.49 gm. (0.0055 mole) of mercuric chloride dissolved in 100 cc. of water. The clear solution gradually developed turbidity while

standing at room temperature for 3 hours. After the mixture had been allowed to stand at 5° for 16 hours, a precipitate had formed which was collected on a sintered glass filter. The precipitate was washed thoroughly with water and then dried *in vacuo* over phosphoric anhydride at room temperature. The light yellow powder weighed 2.67 gm. (94 per cent). The mercury derivative (IIa) was slightly soluble in dioxane, ethanol, and acetone, very slightly soluble in ethyl acetate, and insoluble in water.

$C_{16}H_{17}N_2O_4SClHg$.   Calculated.   N 4.92, S 5.63
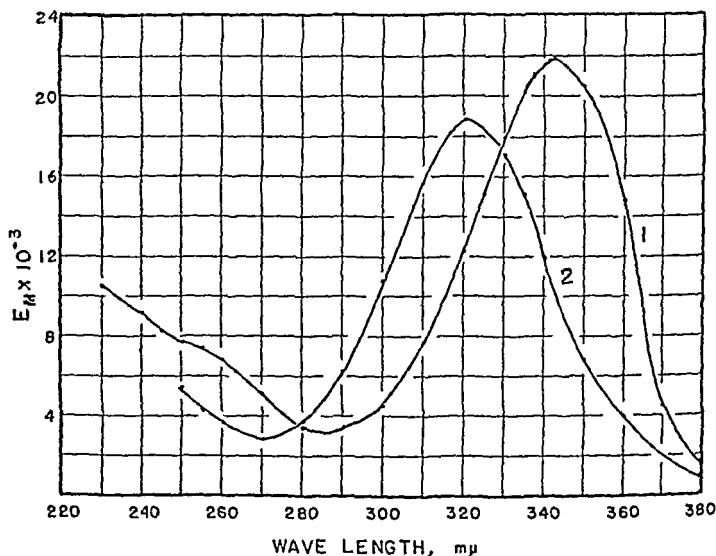569.5   Found.   " 4.86, " 5.40

FIG. 2. Molar absorption spectra[1] of the mercury derivative (IIa) in 95 per cent ethanol (Curve 1) and in dioxane (Curve 2).

The molar absorption spectra of the mercury derivative (IIa) in 95 per cent ethanol (Curve 1) and in dioxane (Curve 2) are shown in Fig. 2.

Conversion of Mercury Derivative (IIa) to Amorphous D-Benzylpenicillenic Acid—A suspension of 2.40 gm. of the mercury derivative (IIa) in 30 cc. of water and 75 cc. of ethyl acetate was treated with hydrogen sulfide. The mixture was centrifuged and the ethyl acetate layer was filtered through diatomaceous earth (Filter-Cel). The filtrate was placed at −70° for 30 minutes to freeze out most of the water. The ice crystals were separated by rapid filtration and washed with 50 cc. of ethyl acetate at −70°. The combined ethyl acetate solutions were allowed to stand for 30 minutes over anhydrous $MgSO_4$. After the desiccant had been separated by gravity filtration, the ethyl acetate solution was concentrated *in vacuo* in the absence of air of ebullition to a volume of 30 cc. The concentrated ethyl

acetate solution was added dropwise to 600 cc. of agitated hexane. The amorphous precipitate which formed turned to a gum when the mixture was allowed to stand for 2 hours at 5°. The supernatant liquid was decanted and the gum was dissolved in 30 cc. of chloroform. Upon dropwise addition of the chloroform solution to 500 cc. of hexane, a white, amorphous precipitate formed. The precipitate was filtered, washed well with hexane, and, while still moist with hexane, placed in a vacuum desiccator to dry under suction. The amorphous D-benzylpenicillenic acid weighed 0.85 gm. (60 per cent). This material possessed an absorption peak in 95 per cent ethanol at 322.5 mμ (E_M = 17,600). The specific rotation determined within 10 minutes after preparing the solution was $[\alpha]_D^{24}$ = +86° (0.47 per cent solution in 95 per cent ethanol).

DL-*Benzylpenicillenic Acid by Admixture of Natural* D- *with Synthetic* L-*Benzylpenicillenic Acid*—Natural D-benzylpenicillenic acid (0.70 gm.) (E_M = 17,600 at 322.5 mμ; $[\alpha]_D^{24}$ = +86°) was admixed with 0.70 gm. of synthetic L-benzylpenicillenic acid (E_M = 17,800 at 322.5 mμ; $[\alpha]_D^{24}$ = −89°). The mixture was treated by a procedure similar to that described above for the isolation of crystalline DL-benzylpenicillenic acid by admixture of the synthetic enantiomorphs. The crystalline DL-benzylpenicillenic acid isolated by this procedure weighed 0.283 gm. (21 per cent), m.p. 133–134° (with decomposition).

A 100 mg. sample of this product was recrystallized from 5 cc. of ethyl acetate. 50 mg. of DL-benzylpenicillenic acid were recovered; m.p. 137–139° (with decomposition); $[\alpha]_D^{23}$ = 0° (0.26 per cent solution in 95 per cent ethanol). There was no depression in the melting point upon admixture with DL-benzylpenicillenic acid prepared from DL-penicillamine (V) and the oxazolone (IV) (7). The molar absorption spectrum in 95 per cent ethanol is shown in Fig. 3.

C$_{16}$H$_{18}$N$_2$O$_4$S.   Calculated.   C 57.45, H 5.43, N 8.38
334.4   Found.   " 57.31, " 5.82, " 8.33

Infra-Red Absorption Measurements—The infra-red absorption spectra from 690 cm.$^{-1}$ to 3600 cm.$^{-1}$ of DL-benzylpenicillenic acid made by admixture of natural D- with synthetic L-benzylpenicillenic acid (upper curve) and of DL-benzylpenicillenic acid made by synthesis from DL-penicillamine (lower curve) are shown in Fig. 4. The absorption spectra were determined on samples of the crystalline compounds mulled in mineral oil between two sodium chloride plates. The measurements were made on a Perkin-Elmer infra-red spectrometer, model 12A, with a gain control to compensate for the energy distribution of the Globar source. It should be pointed out that the curves include absorption peaks due to mineral oil and to air as well as those due to benzylpenicillenic acid.

Rearrangement of Benzylpenicillenic Acid to Benzylpenicillin—Crystalline
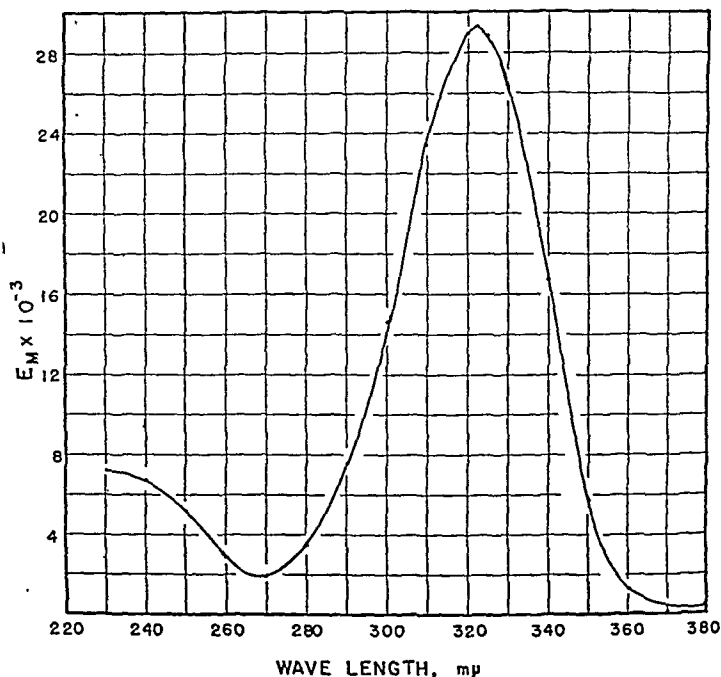
FIG. 3. Molar absorption spectrum[1] of crystalline DL-benzylpenicillenic acid in .5 per cent ethanol.
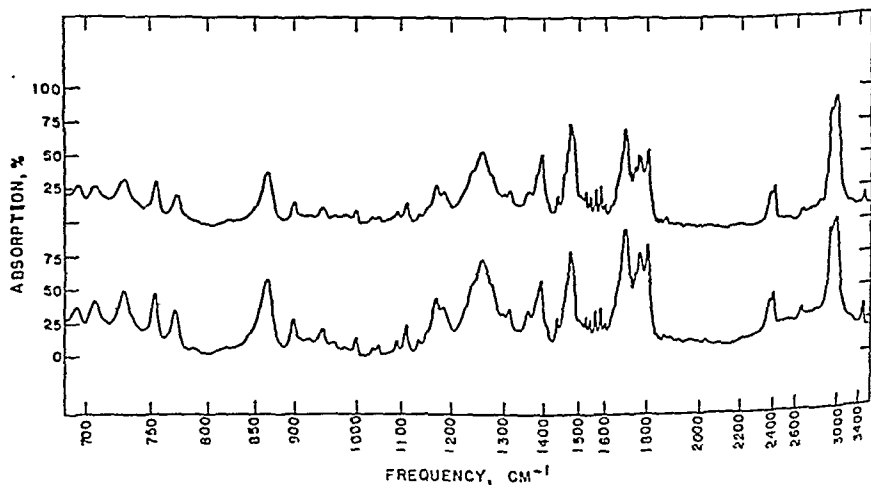

FIG. 4. Infra-red absorption spectra of crystalline DL-benzylpenicillenic acids: upper curve, DL-benzylpenicillenic acid prepared by admixture of natural D- with synthetic L-benzylpenicillenic acid; lower curve, DL-benzylpenicillenic acid synthesized from DL-penicillamine.

DL-benzylpenicillenic acid (30 mg.) prepared by admixture of natural D-
with synthetic L-benzylpenicillenic acid was dissolved in 10 cc. of pyridine
containing 6.5 mg. of pyridinium chloride per cc.   Aliquots (1 cc.) of this
solution were placed in a series of test-tubes.   To one of these tubes about
0.05 cc. of triethylamine was added and the tube was placed in an ice
bath.   The rest of the tubes were placed in an oil bath at 120°.   At noted
time intervals a tube was removed from the oil bath, triethylamine was
added to it, and the tube was cooled in the ice bath.   The solvents were
removed from each tube *in vacuo* at a bath temperature of 50°.   The resi-
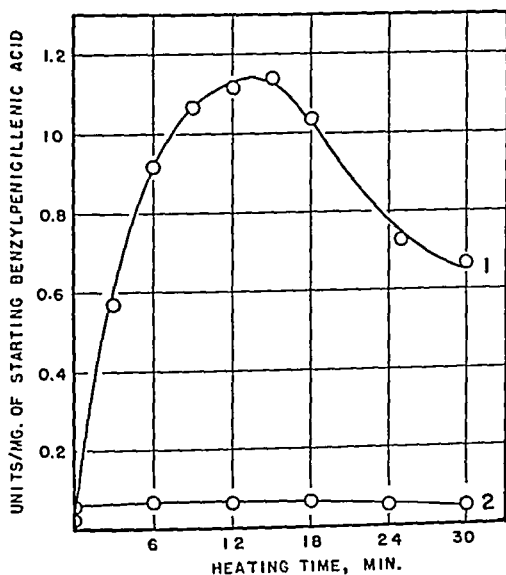
FIG. 5. Antibiotic activity produced by heating benzylpenicillenic acids in pyri-
dine containing pyridinium chloride: Curve 1, crystalline DL-benzylpenicillenic acid
in which the D moiety came from penicillin; Curve 2, synthetic, amorphous L-benzyl-
penicillenic acid.

dues were moistened with 0.2 cc. of acetone and then dissolved in various
amounts of 1 per cent phosphate buffer solution at pH 6.   These buffer solu-
tions were assayed against *Bacillus subtilis* ATCC 6051 by a modification
of the method of Vincent and Vincent (9) with crystalline sodium benzyl-
penicillin as a standard.   The results are shown in Curve 1, Fig. 5.

Synthetic L-benzylpenicillenic acid was heated in pyridine and pyridinium
chloride, and the products were prepared for assay under conditions similar
to those described above.   The results are shown in Curve 2, Fig. 5.   It
should be noted that this crude L-benzylpenicillenic acid possessed a slight

amount of antibiotic activity (about 0.05 unit per mg.) when assayed at high concentration. However, since this antibiotic activity increased only very slightly, if at all, during the heating period, it was probably not due to the presence of a penicillin-like compound.

In another experiment, crystalline DL-benzylpenicillenic acid made by admixture of natural D with synthetic L acid was dissolved at a concentration of 3 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc. An identical solution was prepared from DL-benzylpenicillenic acid made by synthesis from DL-penicillamine (7). The two solutions were placed in an oil bath at 110° for 12 minutes, and then removed and prepared for assay as described above. The DL-benzylpenicillenic acid in which the D moiety arose from penicillin yielded 1.14 units of penicillin per mg. of starting DL-benzylpenicillenic acid, while the entirely synthetic DL-benzylpenicillenic acid gave rise to 1.19 units. Aliquots which had not been heated were also assayed. These showed no detectable activity when assayed at a concentration of 3 mg. per cc.

Rearrangement of Natural D-Benzylpenicillenic Acid to D-Benzylpenillic Acid—A solution of 75 mg. of amorphous, natural D-benzylpenicillenic acid in 1 cc. of methanol was seeded with a trace of D-benzylpenillic acid. The solution was allowed to stand at room temperature for 18 hours and then at 5° for 24 hours. Long, needle-like crystals of D-benzylpenillic acid separated; weight, 10.5 mg. (14 per cent); $[\alpha]_D^{24} = +490°$ (0.1 per cent solution in methanol) (6); $E_M = 6300$ at 237.5 mμ in 95 per cent ethanol (6). The melting point was determined on a sample which had been recrystallized by dissolving it in an equivalent amount of 0.1 N NaOH and then adding an equivalent amount of 0.1 N HCl. This sample of D-benzylpenillic acid melted at 180–185° (with decomposition).[5] The melting point was not lowered upon admixture with D-benzylpenillic acid prepared by rearrangement of benzylpenicillin in water at pH 2 (8).

Rearrangement of DL-Benzylpenicillenic Acid to DL-Benzylpenillic Acid—A methanolic solution (2 cc.) of 100 mg. of crystalline DL-benzylpenicillenic acid in which the D moiety arose from penicillin was seeded with a trace of DL-benzylpenillic acid. After the solution had been allowed to stand for 17 hours at room temperature and 24 hours at 5°, 18.2 mg. (18 per cent) of long, needle-like crystals separated. The DL-benzylpenillic acid had a specific rotation of $[\alpha]_D^{24} = 0°$ (0.1 per cent solution in methanol); $E_M = 5700$ at 240 mμ in 95 per cent ethanol; and m.p. 179–180° (with decomposition).[5] The melting point was not lowered upon admixture with DL-benzylpenillic acid prepared by synthesis from DL-penicillamine through the intermediate DL-benzylpenicillenic acid (7).

[5] In the determination of the melting points of the benzylpenillic acids reported in this paper, the compounds were placed in the bath at 170° and heated at a rate of 1.5° per minute at the melting point.

DL-*Benzylpenillic Acid by Admixture of Enantiomorphs*—D-Benzylpenillic acid and its enantiomorph were prepared from the corresponding D- and L-benzylpenicillenic acids by rearrangement in methanol (6). Each of the penillic acids was dissolved in 0.1 N NaOH solution so that the concentration was 30 mg. per cc. Equal volumes of the two solutions were mixed, and the resulting solution was made acid to Congo red paper with 0.1 N HCl. After the solution had stood at 5° overnight, it yielded white crystals, m.p. 177–178° (with decomposition). The melting point of this DL-benzylpenillic acid was not lowered upon admixture with racemic benzylpenillic acid (m.p. 178.5–179.5°) formed by rearrangement of DL-benzylpenicillenic acid (7).

SUMMARY

D-Benzylpenicillenic acid synthesized from D-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone was shown to be identical with D-benzylpenicillenic acid prepared by rearrangement of D-benzylpenicillin (penicillin G).

Benzylpenicillin was converted to an antibiotically inactive product which was in turn reconverted in small yield to benzylpenicillin. Evidence was presented to show that this inactive compound was identical with D-benzylpenicillenic acid and that D-benzylpenicillenic acid was an intermediate in the synthesis of benzylpenicillin from D-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone.

Racemic benzylpenillic acid prepared by rearrangement of DL-benzylpenicillenic acid was shown to contain a D moiety identical with D-benzylpenillic acid prepared by rearrangement of D-benzylpenicillin.

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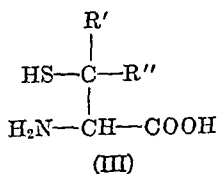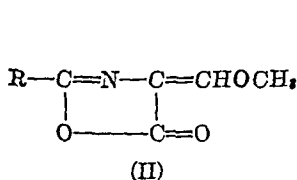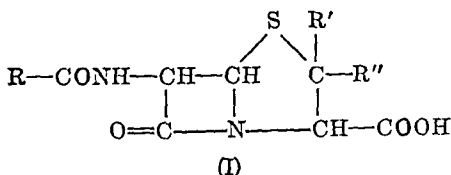9. Vincent, J. G., and Vincent, H. W., *Proc. Soc. Exp. Biol. and Med.,* **55,** 162 (1944).

THE SYNTHESIS OF DL-β,β-DIETHYLCYSTEINE AND DL-β-ETHYL-β-METHYLCYSTEINE

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The various penicillins of natural origin that have so far been isolated (1–4) differ from one another only in the nature of the R group, as illustrated in the general formula (I) based on the β-lactam structure for penicillin. However, the results of chemical studies in this and other laboratories (5, 6) indicate that several penicillins in which various groups have been substituted at R′ and R″ (I) have been synthesized in minute yield, although as yet these substances have not been isolated in pure form. These penicillins were prepared by the condensation of an oxazolone (II) with the appropriate α-amino-β-mercapto acid (III).

$$
\begin{array}{c}
\text{S} \quad \text{R}' \\
\diagup \ \diagdown \ | \\
\text{R—CONH—CH—CH} \quad \text{C—R}'' \\
| \qquad\quad | \qquad\quad | \\
\text{O=C———N———CH—COOH} \\
\text{(I)}
\end{array}
$$

$$
\begin{array}{c}
\text{R—C=N—C=CHOCH}_3 \\
| \qquad\quad | \\
\text{O———C=O} \\
\text{(II)}
\end{array}
\qquad
\begin{array}{c}
\text{R}' \\
| \\
\text{HS—C—R}'' \\
| \\
\text{H}_2\text{N—CH—COOH} \\
\text{(III)}
\end{array}
$$

(IIIa) (R′ = R″ = C_2H_5)
(IIIb) (R′ = C_2H_5, R″ = CH_3)
(IIIc) (R′ = R″ = CH_3)

For a study (7) of further variations of the penicillin molecule at R′ and R″, it was desired to prepare additional α-amino-β-mercapto acids for condensation with an appropriate oxazolone. The synthesis of two such compounds, namely DL-β,β-diethylcysteine (IIIa) and DL-β-ethyl-β-methylcysteine (IIIb), is the subject of the present paper. During the

war-time studies on penicillin, the investigators at the Abbott Laboratories reported the preparation of β-ethyl-β-methylcysteine (IIIb) (8), but did not fully characterize the final product. Consequently the details of its preparation and isolation as the hydrochloride monohydrate (VIIIb) are included here.

The series of reactions used for the synthesis of these two α-amino-β-mercapto acids was similar to that already developed for the synthesis of DL-penicillamine (IIIc) (8).

$$
\begin{array}{c}
\text{R}' \\
\quad \searrow \\
\quad\quad \text{CHCHCOOH} \\
\quad \nearrow \quad | \\
\text{R}'' \quad \text{NH}_2 \\
\text{(IVa,b)}
\end{array}
\xrightarrow[\text{4 N NaOH}]{\text{ClCH}_2\text{COCl}}
\begin{array}{c}
\text{R}' \\
\quad \searrow \\
\quad\quad \text{CHCHCOOH} \\
\quad \nearrow \quad | \\
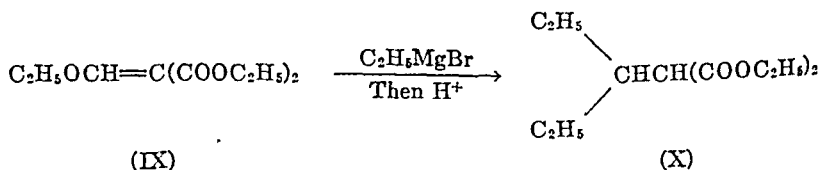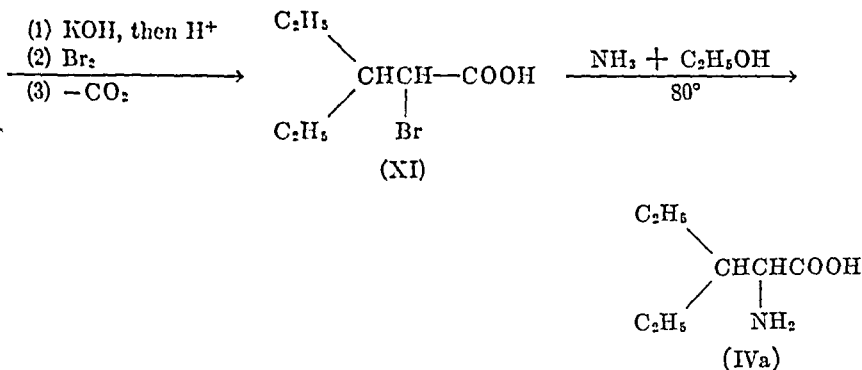\text{R}'' \quad \text{NH}-\text{COCH}_2\text{Cl} \\
\text{(Va,b)}
\end{array}
\xrightarrow{\text{Ac}_2\text{O}}
$$

$$
\begin{array}{c}
\text{R}' \\
\searrow \\
\quad \text{C}=\text{C}-\text{C}=\text{O} \\
\nearrow \qquad | \qquad\;\; | \\
\text{R}'' \qquad\;\; \text{N} \qquad \text{O} \\
\qquad\qquad\quad \searrow \;\; \nearrow \\
\qquad\qquad\quad\;\; \text{C} \\
\qquad\qquad\quad\;\; | \\
\qquad\qquad\quad\; \text{CH}_3 \\
\text{(VIa,b)}
\end{array}
\xrightarrow[\text{Then H}_2\text{O}]{\substack{\text{CH}_3\text{OH} \\ \text{H}_2\text{S}}}
\begin{array}{c}
\text{R}' \\
| \\
\text{R}''-\text{C}-\text{CH}-\text{COOH} \\
| \qquad\;\; | \\
\text{SH} \quad \text{NHCOCH}_3 \\
\text{(VIIa,b)}
\end{array}
\xrightarrow{\text{2 N HCl}}
$$

$$
\begin{array}{c}
\text{R}' \\
| \\
\text{R}''-\text{C}-\text{CH}-\text{COOH} \\
| \qquad\;\; | \\
\text{SH} \quad \text{NH}_2\cdot\text{HCl}\cdot\text{H}_2\text{O} \\
\text{(VIIIa,b)}
\end{array}
$$
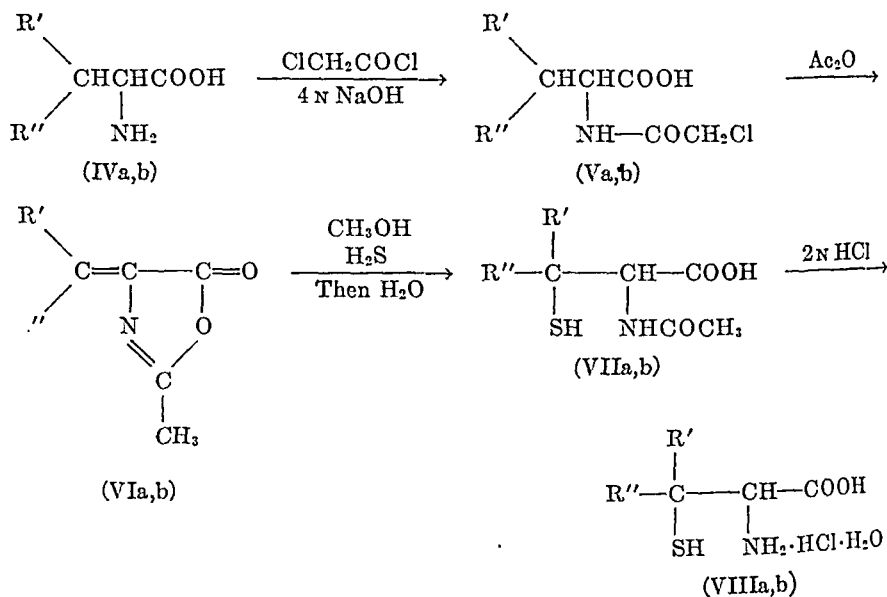
(a) (R' = R'' = C₂H₅); (b) (R' = C₂H₅, R'' = CH₃)

In the above reactions the commercially available DL-isoleucine (IVb) served as a starting compound for the synthesis of DL-β-ethyl-β-methylcysteine hydrochloride monohydrate (VIIIb). However, the DL-β,β-diethylalanine (IVa) needed as a starting compound for the preparation of DL-β,β-diethylcysteine hydrochloride monohydrate (VIIIa) had not been prepared previously. The method devised for the synthesis of the DL-β,β-diethylalanine is outlined in the accompanying equations.

$$
\text{C}_2\text{H}_5\text{OCH}=\text{C}(\text{COOC}_2\text{H}_5)_2
\xrightarrow[\text{Then H}^+]{\text{C}_2\text{H}_5\text{MgBr}}
\begin{array}{c}
\text{C}_2\text{H}_5 \\
\searrow \\
\quad \text{CHCH}(\text{COOC}_2\text{H}_5)_2 \\
\nearrow \\
\text{C}_2\text{H}_5
\end{array}
$$

(IX)   (X)

$$\xrightarrow[\text{(3) } -CO_2]{\substack{\text{(1) KOH, then H}^+ \\ \text{(2) Br}_2}}$$

$$\underset{(XI)}{\overset{C_2H_5}{\underset{C_2H_5}{\diagdown}}\!\!\!\!\!\!\!\!\underset{/}{CH}\underset{\underset{Br}{|}}{CH}\!\!-\!\!COOH} \xrightarrow[80°]{NH_3 + C_2H_5OH}$$

$$\underset{(IVa)}{\overset{C_2H_5}{\underset{C_2H_5}{\diagdown}}\!\!\!\!\!\!\!\!\underset{/}{CH}\underset{\underset{NH_2}{|}}{CH}COOH}$$

Diethyl (1-ethylpropyl)-malonate (X) was prepared in 80 per cent yield by the action of ethyl magnesium bromide with ethoxymethylenemalonic ester (IX) according to the procedure of Reynolds (9). The malonic ester derivative (X) was converted to β,β-diethylalanine (IVa) by the malonic ester synthesis of amino acids (10). In this series of reactions the saponi-fication of the malonic ester, bromination of the resulting acid, and de-carboxylation of the α-bromo acid proceeded smoothly. However, when queous ammonia was used to aminate the α-bromo-β-ethylvaleric acid, hich was not obtained in pure state, difficulty was encountered in the olation of the amino acid from the reaction mixture. This difficulty was rercome by carrying out the amination in ethanolic ammonia.

EXPERIMENTAL[1]

Diethyl (1-Ethylpropyl)-malonate—Ethyl magnesium bromide was pre-pared by the addition of 272 gm. of ethyl bromide in 300 cc. of dry ether to 61 gm. of magnesium in 400 cc. of dry ether. After the addition of the ethyl bromide had been completed, the reaction mixture was heated under gentle reflux. Then 216 gm. of ethoxymethylenemalonic ester in 150 cc. of ether were added over a period of 2 hours. Cooling of the reaction mix-ture in a water bath was necessary during this addition. After the reac-tion mixture had cooled to room temperature, it was poured slowly onto a mixture of 215 cc. of 12 N HCl and 1 kilo of ice. The ether layer was sepa-rated, and the aqueous layer was shaken with three 200 cc. portions of ether. After the combined ether layers had been dried over anhydrous MgSO$_4$, the ether was removed and the residue was distilled. The diethyl (1-ethylpropyl)-malonate distilled at 112–113° and amounted to 183 gm.

[1] All the melting points are corrected and are capillary melting points unless otherwise specified.

or 80 per cent of the theoretical amount based on ethoxymethylenemalonic ester.

α-Bromo-β-ethylvaleric Acid—171 gm. of KOH were dissolved in 150 cc. of water and the solution was heated to 100°. To this solution, diethyl (1-ethylpropyl)-malonate (183 gm.) was added dropwise at first and then more rapidly as the reaction got under way. After the addition was complete, the reaction mixture was heated at 100° with stirring for 5 hours.

The contents of the flask were transferred to a beaker, cooled to 15°, and acidified by the addition of 274 cc. of 12 N HCl. A precipitate which formed after the addition of about 170 cc. of acid disappeared upon addition of the rest of the acid. The aqueous solution was shaken with three 200 cc. portions of ether and the combined ether layers were dried over $CaCl_2$. The ether solution was concentrated to a volume of about 400 cc. and bromine (37.8 cc., 113 gm.) was added. The first 3 to 5 cc. of bromine were added in one portion and the mixture was stirred until the color had disappeared. The remainder of the bromine was added dropwise over a period of about 1 hour. After the addition of bromine was complete, 140 cc. of water were added slowly so as not to produce foaming. The ether layer was separated and the aqueous layer was shaken with a 100 cc. portion of ether. The ether layers were combined, the ether was removed, and the residue was subjected to decarboxylation by heating under a reflux at 140° for 2 hours. The residue was distilled at 5 mm. and the crude α-bromo-β-ethylvaleric acid was collected in two fractions (b.p. 106–125°, 30.1 gm., and b.p. 125–141°, 93.7 gm.). The total weight of these two fractions corresponded to 74 per cent of the theoretical amount based on diethyl (1-ethylpropyl)-malonate.

DL-β,β-*Diethylalanine*—A mixture of 30 gm. of the crude α-bromo-β-ethylvaleric acid (b.p. 125–141°) and 75 cc. of absolute ethanol was cooled to −70° and 45 gm. of liquid ammonia were added. The mixture was heated in an autoclave to 80° over a period of 5 hours. The ethanol and ammonia were then removed by a stream of air and the residue was washed with ether. Although the product consisted of a mixture of the desired amino acid and NH_4Br, it was suitable for our use without further purification. The weight of the mixture amounted to 34.8 gm. When the fraction of α-bromo-β-ethylvaleric acid boiling at 106–125° at 5 mm. was used, a lower yield of product was obtained.

A sample was purified for analysis by recrystallization from 70 per cent ethanol. On the hot stage the crystals of DL-β,β-diethylalanine changed from prisms to needles at 170–185° and melted at 245–249° (micro).

$C_7H_{15}O_2N$.   Calculated.   C 57.9, H 10.41, N 9.65
145.2   Found.   " 57.7, " 10.23, " 9.47

N-Chloroacetyl-DL-β,β-*diethylalanine*—14.4 gm. of crude β,β-diethylalanine were dissolved in 16 cc. of 4 N NaOH and 30 cc. of water. While the solution was cooled in an ice bath, 14.3 gm. of chloroacetyl chloride and 55 cc. of 4 N NaOH were added dropwise with stirring. Then the solution was acidified with 6.3 cc. of 12 N HCl, causing the precipitation of the product. The crude N-chloroacetyl-DL-β,β-diethylalanine (m.p. 122–123.5°) weighed 8.2 gm.

27.0 gm. from several runs were dissolved in 100 cc. of ethanol and 250 cc. of water. The hot solution was treated with 1 gm. of norit and filtered. The N-chloroacetyl-DL-β,β-diethylalanine crystallized from the cooled solution, m.p. 127–129°.

$C_9H_{16}O_3ClN$.   Calculated.   C 48.8, H 7.28, N 6.32
221.7   Found.   " 48.9, " 7.50, " 6.36

2-Methyl-4-(1'-ethylpropylidene)-5(4)-oxazolone—27.2 gm. of recrystallized N-chloroacetyl-β,β-diethylalanine and 40 cc. of acetic anhydride were heated in an oil bath at 60–70° for a period of 2 hours. At the end of this time the acetic anhydride was removed under reduced pressure (water pump) at 60° and the oxazolone was distilled at 54–55° at 0.01 to 0.05 mm. The yield was 11.1 gm. or 54 per cent of the theoretical amount.

For purposes of characterization the oxazolone was converted to α-acetamido-β,β-diethylacrylic acid by heating the oxazolone in an excess of water. The acrylic acid derivative was recrystallized from ethyl acetate to give prisms, m.p. 178–178.5° (with decomposition).

$C_9H_{16}O_3N$.   Calculated.   C 58.4, H 8.16, N 7.57
185.2   Found.   " 58.1, " 8.37, " 7.74

α-Acetamido-β,β-diethylacrylamide was also readily obtained by dissolving 0.2 gm. of the oxazolone in 10 cc. of 10 per cent NH_4OH. After the solution had cooled a crystalline precipitate formed, m.p. 198–214°. This was recrystallized from ethanol, m.p. 220–227°.

$C_9H_{16}O_2N_2$ (184.2).   Calculated, N 15.21; found, N 15.33

N-Acetyl-DL-β,β-*diethylcysteine*—To 48.5 cc. of methanol was added 0.3 gm. of sodium and the resulting solution was saturated with H_2S. 11.0 gm. of 2-methyl-4-(1'-ethylpropylidene)-5(4)-oxazolone were added and H_2S was passed through the solution for 12 hours. The reaction mixture was acidified with 1.1 cc. of 12 N HCl and the methanol was removed by distillation under reduced pressure. The crystalline residue was dissolved in a mixture of 55 cc. of methanol and 225 cc. of water and treated with 1 gm. of charcoal (norit). The N-acetyl-DL-β,β-diethylcysteine (m.p. 158–161°) recovered from the solution weighed 8.9 gm. or 62 per cent of

the theoretical amount. After a sample of the crude compound had been recrystallized twice from aqueous ethanol, it possessed a melting point of 167–168°.

$C_9H_{17}O_3NS$.   Calculated.   C 49.3, H 7.82, S 14.62
219.3   Found.   " 49.3, " 7.80, " 14.93

DL-β,β-*Diethylcysteine Hydrochloride Monohydrate*—7.2 gm. of the N-acetyl-DL-β,β-diethylcysteine were heated under a reflux with 100 cc. of 2 N HCl for 16 hours. After the reaction mixture had been concentrated to a volume of about 30 cc., a crystalline precipitate formed which weighed 2.68 gm., representing 35 per cent of the theoretical amount. A 100 mg. sample was recrystallized twice from 5 cc. of 12 N HCl. The twice recrystallized material was dissolved in 0.2 cc. of absolute ethanol and to this were added 2 cc. of absolute ether. The crystals thus obtained had a capillary melting point of 126–127°. However, when the crystals were heated slowly on the hot stage, they changed from prisms to needles at 152° and melted at 176–177° (micro).

$C_7H_{15}O_2NS \cdot HCl \cdot H_2O$.   Calculated.   C 36.3, H 7.83, Cl 15.32
231.7   Found.   " 36.4, " 7.97, " 15.38

2-Methyl-4-(sec-butylidene)-5(4)-oxazolone—A mixture of 69.6 gm. of N-chloroacetyl-DL-isoleucine (11) and 110 cc. of acetic anhydride was agitated by a stream of nitrogen while being heated at 55–60° for 2 hours. After the acetic anhydride had been removed by distillation under reduced pressure (water pump), the residue was distilled at 0.01 to 0.2 mm. and the product distilling at 50–61° was collected. The yield of oxazolone was 41.0 gm. or 79 per cent of the theoretical amount. It was found advisable to use this material immediately for the preparation of N-acetyl-DL-β-ethyl-β-methylcysteine.

The oxazolone was characterized by conversion to α-acetamido-β-ethyl-β-methylacrylic acid (m.p. 174–175°) by heating the oxazolone in an excess of water.

$C_8H_{13}O_3N$.   Calculated.   C 56.1, H 7.65, N 8.18
171.2   Found.   " 56.2, " 7.68, " 8.01

N-Acetyl-DL-β-*ethyl*-β-*methylcysteine*—1.15 gm. of sodium were dissolved in 190 cc. of methanol and the resulting solution was saturated with H_2S. 41 gm. of 2-methyl-4-(sec-butylidene)-5(4)-oxazolone were dissolved in 55 cc. of methanol and this solution was added to the sodium methylate solution. H_2S was passed through the reaction mixture for 16 hours. The mixture was acidified with 4.5 cc. of 12 N HCl and the methanol was removed by distillation under reduced pressure. The residue was crystal-

lized from a mixture of 150 cc. of water and 5 cc. of methanol. This crude product (m.p. 138–138.5°) weighed 44.1 gm. or 80 per cent of the theoretical amount. After the crude product had been treated with charcoal (norit) and crystallized from water, the N-acetyl-DL-β-ethyl-β-methylcysteine possessed a melting point of 144–146.5° and was suitable for conversion to the amino acid. A sample prepared for analysis by two recrystallizations from water had a melting point of 144–145°.

$C_8H_{15}O_3NS$.   Calculated.   C 46.8, H 7.37, S 15.62
205.3   Found.   " 46.6, " 7.44, " 15.86

DL-β-Ethyl-β-methylcysteine Hydrochloride Monohydrate—6.1 gm. of the recrystallized N-acetyl-β-ethyl-β-methylcysteine were heated under a reflux with 85 cc. of 2 N HCl for 16 hours. The volume of the reaction mixture was concentrated to about 20 cc. and the crystalline product was collected. The yield amounted to 2.41 gm. or 38 per cent of the theoretical amount. The β-ethyl-β-methylcysteine hydrochloride monohydrate was purified by recrystallization from 12 N HCl. As in the case of the β,β-diethylcysteine hydrochloride monohydrate, a difference was noted between the capillary melting point and that obtained on the hot stage. The capillary melting point was 117–119°, while that obtained on the hot stage was 169–170° (micro).

$C_6H_{13}O_2NS \cdot HCl \cdot H_2O$.   Calculated.   C 33.1, H 7.41, Cl 16.29
217.7   Found.   " 33.1, " 7.74, " 16.29

SUMMARY

The synthesis of DL-β,β-diethylalanine and its use in the preparation of DL-β,β-diethylcysteine hydrochloride monohydrate have been described. In addition, details have been presented for the synthesis of DL-β-ethyl-β-methylcysteine hydrochloride monohydrate from DL-isoleucine.

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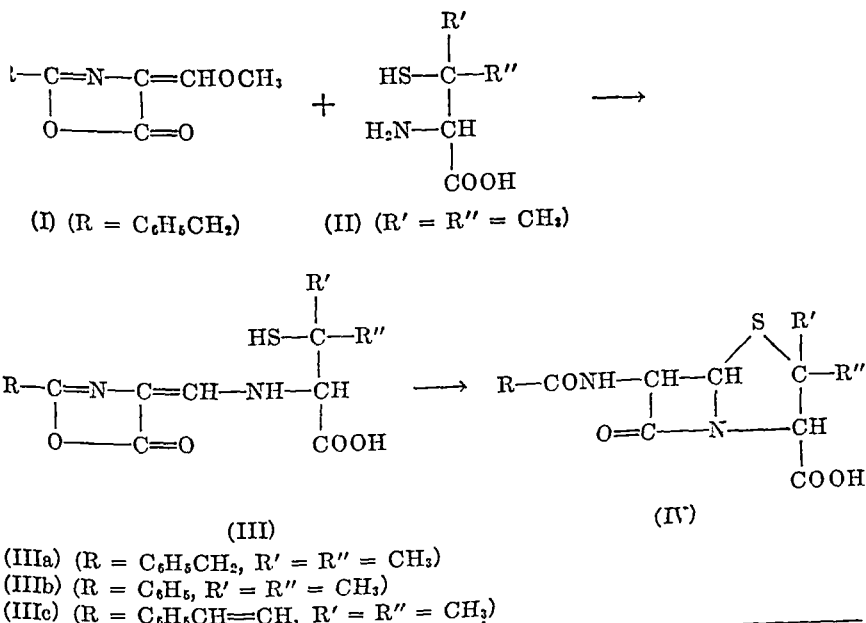
THE PREPARATION AND ANTIBACTERIAL PROPERTIES OF THE CRUDE SODIUM SALTS OF SOME SYNTHETIC PENICILLINS*

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DOROTHY S. GENGHOF, ARTHUR H. LIVERMORE,
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In previous communications (1–3) it has been demonstrated that the antibiotic activity (4) produced by heating D-penicillamine (II) hydrochloride hydrate with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) in pyridine is due to the synthesis of benzylpenicillin. Because of these results there can be little doubt that, when oxazolones substituted with other groups in the 2 position are condensed with penicillamine hydrochloride in pyridine, the antibiotic activity (1, 4) produced is due to the synthesis of penicillins differing from benzylpenicillin in the nature of the

(I) (R = C₆H₅CH₂)   (II) (R′ = R″ = CH₃)

(III)   (IV)

(IIIa) (R = C₆H₅CH₂, R′ = R″ = CH₃)
(IIIb) (R = C₆H₅, R′ = R″ = CH₃)
(IIIc) (R = C₆H₅CH=CH, R′ = R″ = CH₃)

R group, as illustrated on the basis of the β-lactam structure (IV). Moreover, the antibiotic activity (1, 4) produced by the condensation of the oxazolone (I) with α-amino-β-mercapto acids other than D-penicillamine must be due to the synthesis of analogues of penicillin (IV) differing from the known varieties in the nature of the groups R′ and R″.

The results of studies (3, 5, 6) on the mechanism of synthesis of benzylpenicillin from 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) and D-penicillamine (II) have proved beyond reasonable doubt that D-benzylpenicillenic acid (IIIa) is an intermediate in the synthesis of benzylpenicillin. Since these studies were hindered by difficulties in obtaining this intermediate compound in crystalline form, it seemed advisable to introduce other oxazolones or other α-amino-β-mercapto acids into the investigation with the thought in mind that their use might result in the production of crystalline intermediate compounds. Some justification for this reasoning was afforded by the finding that the substitution of DL-penicillamine for D-penicillamine in the condensation with the oxazolone (I) in pyridine containing triethylamine resulted in the production of a crystalline intermediate compound, identified as DL-benzylpenicillenic acid (IIIa) (5, 6).

Although the mold produces a number of different penicillins (7), especially in the presence of specific precursors (8, 9), all of the penicillins thus far isolated from the mold cultures have contained the D-penicillamine moiety and have differed from one another only in the nature of the R group (IV). It was of interest, therefore, to prepare penicillins in which the D-penicillamine moiety was replaced by other α-amino-β-mercapto acids. Penicillins of this type could be prepared in crude form by the substitution of various α-amino-β-mercapto acids for D-penicillamine in the synthetic reaction. These crude penicillins could then be subjected to biological testing to determine whether or not the antibacterial properties of penicillin could be markedly changed by the replacement of the penicillamine moiety with various α-amino-β-mercapto acids.

In the present communication a report is made of the synthesis and antibacterial properties of penicillins obtained by condensation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) (10) with each of the following α-amino-β-mercapto acids: DL-β-methylcysteine, Isomer A (11); DL-β-methylcysteine, Isomer B (11); DL-β,β-diethylcysteine hydrochloride hydrate (12); and DL-β-ethyl-β-methylcysteine hydrochloride hydrate (12, 13). In addition, the synthesis and antibiotic activities of penicillins produced by condensation of D-penicillamine (II) hydrochloride hydrate (13) with either 2-phenyl-4-ethoxymethylene-5(4)-oxazolone (4, 10) or 2-styryl-4-ethoxymethylene-5(4)-oxazolone (4, 10) were studied.

The two-step reaction (1, 3) was used for the synthesis of these penicillins. In the first step the oxazolone was condensed with the α-amino-β-

mercapto acid in aqueous pyridine containing triethylamine to give the intermediate penicillenic acid (III). In only one instance, the case of the D-styrylpenicillenic acid (IIIc), was the penicillenic acid obtained in crystalline form. In all the other cases, the penicillenic acids were obtained in the form of amorphous solids. The ultraviolet absorption spectra of the various penicillenic acids were determined. It was found (Table I) that all of the penicillenic acids made by condensation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone (I) with various α-amino-β-mercapto acids possessed nearly identical absorption curves which, like that of crystalline DL-benzylpenicillenic acid (IIIa) (5, 6), had their main absorption peak at about 320 mμ. On the other hand, the D-phenylpenicillenic acid (IIIb) had its main absorption peak at 355 mμ, while the crystalline D-styrylpenicillenic acid (IIIc) had its main peak at 374 mμ (Fig. 1).

In the second step of the synthesis, the various penicillenic acids (III) were converted in small yield to the corresponding penicillins by heating the penicillenic acids at a concentration of 100 mg. per cc. in pyridine containing pyridinium chloride. Preliminary experiments were performed to determine the optimum heating time for obtaining the maximum yield of activity from the rearrangement of each penicillenic acid. An optimum time of 9 minutes was found for all of the penicillenic acids in which R′ and R″ (III) were both alkyl groups (Table I). However, under the same conditions the optimum heating time was increased to about 20 minutes when one alkyl group at either R′ or R″ was replaced by hydrogen. On the other hand, the substitution of various groups at R (III) did not appear to affect the optimum heating time. It should be pointed out in this connection that the optimum heating time for the rearrangement of a particular penicillenic acid is dependent on the concentration of the penicillenic acid as well as the concentration of the hydrogen chloride in the pyridine (3).

The crude products, containing minute amounts of the various penicillins, were converted to their sodium salts for biological testing. These sodium salts, obtained as amorphous solids, possessed an antibiotic activity of 0.015 to 0.50 unit per mg., depending upon the particular penicillin, when assayed against *Staphylococcus aureus* H with crystalline sodium benzylpenicillin as a standard. The antibiotic activity of each penicillin was also determined quantitatively on two other organisms, *Bacillus subtilis* (ATCC 6051) and *Vibrio metchnikovii* (ATCC 7708). The ratios of the antibiotic activities found on each of these two organisms to that found on *Staphylococcus aureus* were calculated (Table I). It is interesting to note that each of the new synthetic penicillins could be distinguished from each other by the differences in their effect on these three organisms.

The synthetic penicillins were also tested for antibiotic activity against

TABLE I

Preparation of Crude Penicillenic Acids and Corresponding Sodium Penicillins

5(4)-Oxazolones	α-Amino-β-mercapto acids	Yield	Wave-length	$E_M \times 10^{-3}$*	Optimum heating time	S. aureus	B. subtilis / S. aureus	V. metchnikovi / S. aureus
		per cent	mμ		min.	units per mg.†		
2-Benzyl-4-methoxymethylene-	DL-β-Methyl-cysteine, Isomer A	68	320 240	18.6 4.5	20	0.26	0.82	0.45
"	DL-β-Methyl-cysteine, Isomer B	93	320 240	18.7 4.1	20	0.11	1.09	1.91
"	DL-β,β-Diethyl-cysteine·HCl·H$_2$O	86	320	14.7	9	0.42	1.13	0.58
"	DL-β-Ethyl-β-methylcysteine·HCl·H$_2$O	45	320	13.1	9	0.47	1.14	1.27
"	D-Penicillamine-HCl·H$_2$O	75	320 240	20.0 5.1	9			
2-Phenyl-4-ethoxymethylene-	D-Penicillamine-HCl·H$_2$O	95	355 285 240	29.0 6.2 10.5	9	0.018	0.72	5.17
2-Styryl-4-ethoxymethylene-	D-Penicillamine-HCl·H$_2$O	98	374 280	40.0 13.0	9	0.014	0.50	0.96

* E_M is the molar absorption coefficient and is equal to D/cl where D is log I_0/I, c is the concentration in moles per liter, and l is the thickness of cell in cm.

† Experiments (1, 4, 6) with D-, L-, and DL-penicillamine have shown that only the D form gives rise to antibiotic activity in the synthetic reaction. Therefore one would expect that the antibiotic activity produced by the racemic α-amino-β-mercapto acids used here would represent only one-half the activity obtainable from the pure D forms.

seven microorganisms which were resistant to the action of benzylpenicillin. None of the crude sodium penicillins when tested at a concentration of 20 mg. per cc. (0.3 to 10 *Staphylococcus aureus* units per cc., depending on the

penicillin) inhibited the growth of these particular organisms.   Therefore it appears that substitution of the methyl groups of benzylpenicillin at R′ and R″ (IV) by various other groups did not appreciably change the action of penicillin on the resistant bacteria.   Also, the crude synthetic penicillins in which the benzyl group of benzylpenicillin at R (IV) was replaced by a phenyl or styryl group did not show marked antibiotic activity against the seven resistant organisms.

The isolation of D-styrylpenicillenic acid in crystalline form made it possible to corroborate other evidence (5, 6) that penicillenic acid is an
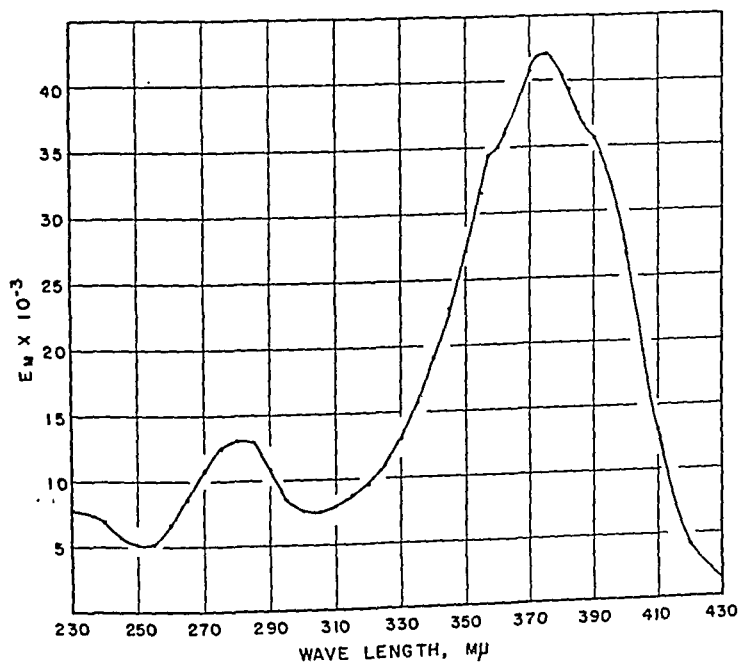

FIG. 1. Molar absorption spectrum of D-styrylpenicillenic acid in 95 per cent ethanol.

intermediate in the synthesis of penicillin from the condensation of various oxazolones with α-amino-β-mercapto acids.   The ability of D-styryl-penicillenic acid (IIIc) to produce a small amount of antibiotic activity upon heating it in pyridine containing pyridinium chloride was retained through several crystallizations of the compound.   Furthermore, it was possible to demonstrate (Table II) that various fractions obtained from the recrystallization of D-styrylpenicillenic acid (IIIc) gave rise to antibiotic activity in an amount proportional to the D-styrylpenicillenic acid present in these fractions.

EXPERIMENTAL

Preparation of Penicillenic Acids—0.011 mole of the α-amino-β-mercapto acid hydrochloride hydrate was dissolved in 2.5 cc. of water or, if the free α-amino-β-mercapto acid was used, 0.011 mole of the compound was dissolved in 2.5 cc. of water containing an equivalent amount of HCl. The aqueous solution was cooled in an ice bath. The oxazolone (0.0102 mole) was dissolved in 10 cc. of pyridine, the solution was cooled to 0°, and 2.5 cc. of redistilled triethylamine were added. (In the case of DL-β,β-diethylcysteine and of DL-β-ethyl-β-methylcysteine, it was found necessary to use approximately 3 times as much water to dissolve these amino acids. Therefore, in the preparation of the oxazolone solutions, the volume of pyridine was increased in a proportional amount.) The cold oxazolone solution was added to the cold solution of the α-amino-β-mercapto acid. After the resulting mixture had been allowed to stand for 10 minutes in an ice bath, it was diluted with 125 cc. of ice-cold chloroform. The resulting mixture was immediately shaken for 1 minute with 125 cc. of ice-cold 2 M H_3PO_4. The chloroform layer was dried at 0° over anhydrous Na_2SO_4 and was then concentrated to dryness *in vacuo* in the absence of air of ebullition at a bath temperature of 30°.

The residue was dissolved in 22 cc. of chloroform and the solution was added dropwise to 450 cc. of agitated hexane. The resulting precipitate of a penicillenic acid was collected on a filter, washed well with hexane, and, while still moist with hexane, placed in a vacuum desiccator to dry under suction. If the resulting amorphous penicillenic acid was not used immediately, it was stored under anhydrous conditions at −10°. The yields of crude penicillenic acids, calculated on the basis of the amount of oxazolone used, and the positions of the absorption peaks in the ultraviolet region are recorded in Table I.

Crystalline D-*Styrylpenicillenic Acid*—D-Penicillamine hydrochloride hydrate (2.24 gm.) and 2.5 gm. of 2-styryl-4-ethoxymethylene-5(4)-oxazolone were condensed under conditions similar to those described above. When the dried chloroform solution of D-styrylpenicillenic acid was concentrated to 25 cc., a crystalline precipitate separated. The crude crystalline D-styrylpenicillenic acid weighed 1.55 gm., representing 45 per cent of the theoretical amount from the starting oxazolone, and possessed a melting point of 127–130° (with decomposition). After two recrystallizations from chloroform-hexane, the yellow crystals of the D-styrylpenicillenic acid melted at 146.5–148° (with decomposition) and had a specific rotation of $[\alpha]_D^{21} = -517°$ for a 0.3 per cent solution in chloroform.

$$C_{17}H_{18}O_4N_2S. \quad \text{Calculated.} \quad C\ 58.9,\ H\ 5.24$$
$$346.4 \quad\quad\quad\ \text{Found.} \quad\quad\text{``}\ 58.5,\ \text{``}\ 5.54$$

The molar absorption curve in the ultraviolet region of D-styrylpenicillenic acid in 95 per cent ethanol is shown in Fig. 1.

Determination of Optimum Heating Time for Conversion of Penicillenic Acids to Penicillins—The penicillenic acid was dissolved so as to give a solution with a concentration of 100 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc.   An aliquot (0.1 to 0.2 cc.) was removed and added to about 0.05 cc. of triethylamine, and the resulting solution was cooled in an ice bath.   The remainder of the original solution was placed in an oil bath preheated to 130°.   At 3 or 4 minute intervals aliquots were removed, added to triethylamine, and cooled in an ice bath.   The solvents were removed *in vacuo* from each aliquot in a water bath at 50°. The residues were moistened with 0.2 cc. of acetone and then dissolved in various amounts of 1 M phosphate buffer solution at pH 7.   These buffer solutions were assayed against *Staphylococcus aureus* H by an agar diffusion method (14, 15) with crystalline sodium benzylpenicillin as a standard. The optimum heating times as determined by this method for the rearrangement of the various penicillenic acids are shown in Table I.   The curves obtained by plotting the yield of activity against the heating time for D-styrylpenicillenic acid (broken line) and for the penicillenic acid made by the condensation of Isomer A of DL-β-methylcysteine with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (solid line) are shown in Fig. 2.

Preparation of Crude Sodium Penicillins for Biological Testing—The penicillenic acids were rearranged in hot pyridine-pyridinium chloride in amounts no larger than 1 gm.   The penicillenic acid (1 gm.) was dissolved in 10 cc. of pyridine containing 6.5 mg. of pyridinium chloride per cc.   The resulting solution, contained in a 20 × 150 mm. test-tube, was placed in a large oil bath preheated to 130°.   After the pyridine began to boil, the position of the tube in the bath was adjusted so that the pyridine refluxed gently from the sides of the tube.   The reaction mixture was kept in the oil bath for the optimum length of time, as given in Table I.   At the end of the heating period, 1 cc. of triethylamine was added to the reaction mixture and the resulting solution was cooled in an ice bath.

Two of the 1 gm. reaction mixtures were combined and the pyridine was removed under reduced pressure at 50° in such a manner that it took no longer than 8 minutes to concentrate the combined solutions to a gummy residue.   The residue from 2 gm. of starting penicillenic acid was dissolved in 100 cc. of ice-cold chloroform and the resulting solution was shaken with 100 cc. of ice-cold 2 M phosphate buffer at pH 1.6 (prepared by admixture of equal volumes of 2 M H_3PO_4 and 2 M NaH_2PO_4).   The chloroform layer was dried over anhydrous Na_2SO_4 at 0° and filtered from the desiccant, and the filtrate was diluted to 150 cc. with chloroform.   A small amount of 1 N NaOH was added to 150 cc. of water and this solution was shaken with

the chloroform solution. The pH of the aqueous layer was then determined. Additional alkali was added in discrete portions and the shaking with the chloroform solution was repeated until the pH of the aqueous layer reached 7 to 7.5. This procedure generally caused emulsions to form which were broken by centrifugation. The aqueous layer was immediately frozen and the water was removed by lyophilization. The resulting amorphous sodium salt was dried to constant weight in a vacuum desiccator over P_2O_5. This sodium salt which contained small amounts of the synthetic sodium penicillin was used for the biological testing. The weights of the
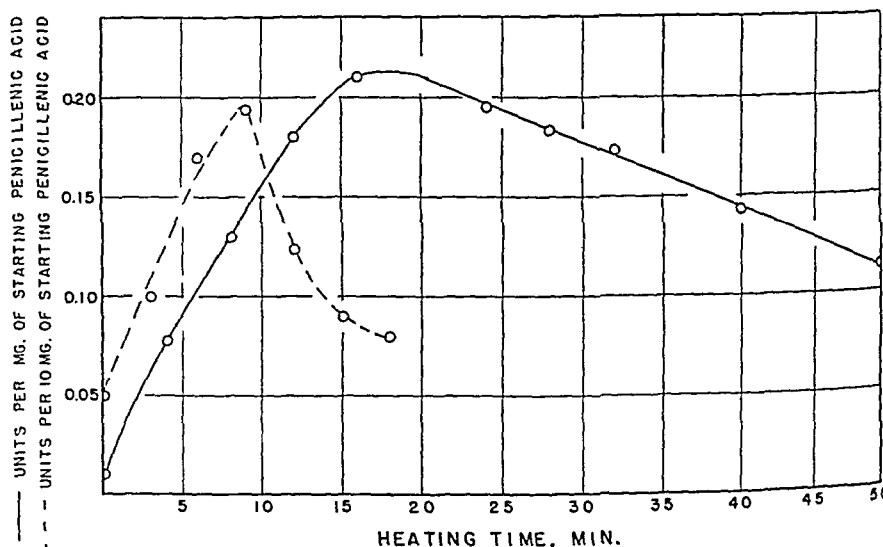

FIG. 2. Production of antibiotic activity on heating penicillenic acids in pyridine containing pyridinium chloride: penicillenic acid produced by the condensation of DL-β-methylcysteine, Isomer A, with 2-benzyl-4-methoxymethylene-5(4)-oxazolone (solid line); D-styrylpenicillenic acid (broken line).

crude sodium salts ranged from 50 to 70 per cent of the weights of the penicillenic acids used as starting material.

Antibiotic Activity of Crude Synthetic Sodium Penicillins; Quantitative— The relative activities of the sodium salts of the crude synthetic penicillins were determined quantitatively against three microorganisms, *Staphylococcus aureus* H, *Bacillus subtilis* (ATCC 6051) and *Vibrio metchnikovii* (ATCC 7708). The activities were determined by an agar diffusion method with filter paper disks (Schleicher and Schüll, No. 740-E) (14, 15). Crystalline sodium benzylpenicillin was used as a standard in measuring the activities of each preparation against each organism. The assays involving the use of *Bacillus subtilis* and *Staphylococcus aureus* were essentially

similar to those described by Foster and Woodruff (16) and Schmidt and Moyer (17) for these two organisms, with the exception that filter paper disks were used instead of cylinders.

The method developed for use with *Vibrio metchnikovii* was similar to that employing *Staphylococcus aureus* (17). In this assay, brain-heart infusion agar was used. First a layer of unseeded agar (12 cc.) was poured into sterile Petri dishes (100 mm. in diameter) with unglazed porcelain covers (Coors). After the agar had hardened, a layer of seeded agar (4 cc.) was added to each plate. Care was exercised to prevent the temperature of the agar seeded with *V. metchnikovii* from exceeding 45°. In order to prepare the inoculum, a slant of *V. metchnikovii* on infusion agar contained in a 15 × 150 mm. test-tube was incubated at 37° for 18 hours. The cells were washed from the slant with 5 cc. of sterile saline solution and 0.1 cc. of this suspension was used for every 4 cc. of agar. After the standard and unknown solutions had been added, the plates were incubated overnight at 37° and the diameters of the zones of inhibition of growth were measured. Since *V. metchnikovii* is less sensitive than *Staphylococcus aureus* to benzylpenicillin, the standard curve was determined by points ranging from 1 to 8 units of benzylpenicillin per cc. As the crude mixtures containing synthetic penicillin appeared to react gradually with water liberating an acidic group, it was necessary to use a strong buffer to prevent a drop in pH in solutions containing high concentrations of the test compounds. Therefore 1 M phosphate buffer at pH 7.0 was used to dissolve the standard sodium benzylpenicillin and the unknown samples. The results obtained on the assay of the crude mixtures containing synthetic penicillins are shown in Table I.

Qualitative—A study was made of the effect of the crude mixtures containing synthetic penicillins on the growth of seven organisms that were resistant to the action of benzylpenicillin. The organisms used were as follows: *Aerobacter aerogenes* (ATCC 8308), *Klebsiella pneumoniae* (ATCC 9997), *Mycobacterium smegmatis* (ATCC 101), *Mycobacterium tuberculosis* (ATCC 607), *Escherichia coli*, *Proteus vulgaris* OX-19, and *Pseudomonas aeruginosa*. The last three organisms were obtained from Professor James M. Neill.

Known amounts of the various synthetic reaction mixtures were placed in sterile Petri dishes. The crude sodium salt was dissolved in 1 cc. of 1 M phosphate buffer solution at pH 7.0, 9 cc. of brain-heart infusion agar (1.8 per cent) at 45° were added, and the resulting solution was mixed thoroughly. Each synthetic reaction mixture was tested at levels of 5, 10, and 20 mg. per cc. of agar. After the agar had hardened, each plate was streaked in a single line with a loopful of each of the seven organisms grown in broth. The plates were then incubated at 37° for 16 to 18 hours and

read by macroscopic examination to see whether or not growth had occurred. The following control plates were prepared and used with each experiment: plates containing 1 cc. of buffer solution but no added penicillin, plates to which 1 cc. of buffer solutions (1 M phosphate at pH 7.0) of various concentrations of crystalline sodium benzylpenicillin was added so that the final plates contained 1 to 100 units (0.6 to 60 γ) of sodium benzylpenicillin per cc. of agar, and plates containing 5, 10, and 20 mg. (or about 5, 10, and 20 units) of crude synthetic sodium benzylpenicillin per cc. of agar. The broth cultures of the organisms used to streak the plates were prepared as follows: *Proteus vulgaris* was grown for 2 days in brain-heart infusion broth, the two *Mycobacteria* were grown for 2 days in Dubos broth (18), and all the other organisms were grown for 1 day in nutrient broth.

The results of the above experiments indicated that the growth of the organisms, with the possible exception of the *Mycobacteria* and the *Proteus*, was not inhibited by any of the synthetic penicillins when the crude reaction mixtures were tested at levels up to 20 mg. per cc. of agar. Although the growth of *Mycobacteria* and *Proteus* was not inhibited by 100 units (60 γ) of crystalline sodium benzylpenicillin, their growth was inhibited by the plates containing 20 mg. of a crude synthetic benzylpenicillin preparation per cc. of agar and by similar quantities of crude preparations of the other synthetic penicillins. Since the crude synthetic benzylpenicillin preparation contained only about 1 unit per mg., it was obvious that the growth of the organisms was being inhibited by substances in the reaction mixture other than penicillin. Further evidence on this point was obtained when the synthetic penicillins were assayed against *Mycobacterium tuberculosis* by an agar diffusion method with filter paper disks. The assay was similar to that described above for *Vibrio metchnikovii* with the exception that *Mycobacterium tuberculosis* was used as the test organism, and the Dubos medium (18) containing 1.5 per cent agar was used in place of the brain-heart infusion agar. In this assay, none of the crude synthetic penicillins including the crude sodium benzylpenicillin produced a zone of inhibition when tested at a concentration of 20 mg. per cc. Under the same conditions, 10 units of streptomycin per cc. produced a zone of inhibition measuring 21 mm. in diameter.

Fractional Crystallization of D-*Styrylpenicillenic Acid and Conversion of Fractions to Crude* D-*Styrylpenicillin*—600 mg. of D-styrylpenicillenic acid (Fraction A) were dissolved in 60 cc. of hot chloroform and 60 cc. of hot hexane were added. After the solution had cooled, 220 mg. of crystalline D-styrylpenicillenic acid (Fraction B) separated. Concentration of the mother liquor to dryness gave 237 mg. of residue (Fraction C). 180 mg. of Fraction B were recrystallized from 25 cc. of chloroform plus 21 cc. of

hexane.   The recrystallized product (Fraction D) weighed 55 mg.   Concentration of the mother liquor to dryness gave 115 mg. of residue (Fraction E).   Aliquots were removed from each fraction for the determination of melting point and molar absorption at 375 mμ in 95 per cent ethanol, and for conversion to crude D-styrylpenicillin (Table II).

In order to convert D-styrylpenicillenic acid to D-styrylpenicillin, the fractions of the D-styrylpenicillenic acid were dissolved so as to give solutions with a concentration of 10 mg. per cc. in pyridine containing 6.5 mg. of pyridinium chloride per cc.   The resulting solutions were all placed at the same time in an oil bath at 130°.   After 15 minutes the solutions were removed from the bath, 0.10 cc. of triethylamine was added per cc. of solution, and the resulting solutions were cooled in an ice bath.   The solvents were removed *in vacuo*, and the residues were dissolved in various amounts of 1 per cent phosphate buffer at pH 7.0 and assayed against *Staphylococcus*

TABLE II

Properties of Fractions from Recrystallization of D-Styrylpenicillenic Acid

Fraction	Melting point (decomposition)	E_M at 375 mμ (a)	Antibiotic activity produced (b)	$\frac{(b)}{(a)} \times 10^7$
	°C.		*unit per mg. starting fraction*	
A	127–130	37,400	0.0175	4.68
B	146–148	42,300	0.0190	4.50
C	112–121	25,250	0.0125	4.95
D	146–148	42,300	0.0195	4.61
E	122–127	35,700	0.0160	4.48

aureus with crystalline sodium benzylpenicillin as the standard.   For each fraction, the ratio of antibiotic activity produced to molar absorption at 375 mμ was calculated (Table II).   These ratios remained quite constant, ranging from 4.48×10^{-7} to 4.95×10^{-7}.

SUMMARY

The synthesis in minute yields and the preparation in the form of crude sodium salts of several penicillins have been described.   Some of the penicillins were analogues of benzylpenicillin in which the D-penicillamine moi-

ety of benzylpenicillin was replaced by either DL-β-methylcysteine, Isomer A; DL-β-methylcysteine, Isomer B; DL-β,β-diethylcysteine; or DL-β-ethyl-β-methylcysteine. In addition crude sodium D-phenylpenicillin and D-styrylpenicillin were synthesized.

In the first step of the two-step reaction used in the synthesis of these penicillins, appropriate oxazolones were condensed with various α-amino-β-mercapto acids to yield penicillenic acids. In most cases, the penicillenic acids were isolated as amorphous solids which were characterized by their ultraviolet absorption spectra. In the case of D-styrylpenicillenic acid, the intermediate penicillenic acid was obtained in crystalline form. In the second step of the synthesis, the penicillenic acids were converted in small yield to the corresponding penicillins. It was found that the optimum conditions for this rearrangement were dependent among other things upon the type of penicillenic acid involved.

The relative antibiotic activities of the crude preparations of the synthetic penicillins were determined on three microorganisms: *Staphylococcus aureus* H, *Bacillus subtilis*, and *Vibrio metchnikovii*. In qualitative tests it was found that the crude synthetic penicillins did not possess marked antibacterial properties against *Aerobacter aerogenes*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Proteus vulgaris* OX-19, or *Pseudomonas aeruginosa*. These organisms also showed a high degree of resistance to the action of crystalline sodium benzylpenicillin.

Studies on crystalline D-styrylpenicillenic acid have added to the evidence that penicillenic acids are intermediates in the synthesis of penicillins in the reaction of appropriate oxazolones with α-amino-β-mercapto acids.

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STUDY OF CARBON DIOXIDE FIXATION IN THE
SYNTHESIS OF CITRULLINE*

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The ornithine → citrulline → arginine cycle of urea synthesis originally proposed by Krebs and Henseleit (1) has had increasing experimental support (2–10). The position of citrulline in the cycle, however, has been questioned (11). With the advent of the successful separation of the two over-all enzymatic steps, ornithine → citrulline and citrulline → arginine (10), from the intact cellular system the position of citrulline appeared to be well established. However, it seemed desirable to determine the intermediary rôle of citrulline by the use of carbon dioxide containing C^{14}. In the present paper it is demonstrated that the incorporation of C^{14} into the carbonyl group of citrulline and urea is of such a magnitude that citrulline must be considered as an obligatory intermediate in the urea synthesis cycle.

Procedures

Tissue Preparations—The enzyme preparations used in this study were the KCl-washed rat liver residue for the step ornithine → citrulline (10) and whole liver homogenate for the step citrulline → urea, both previously described by Cohen and Hayano (9).

Substrates—L-Ornithine and L-citrulline were prepared from L-arginine according to the method of Hunter (12) and Gornall and Hunter (13). Adenosine triphosphate (ATP) was prepared from rabbit muscle (14).

Analytical—Citrulline was estimated by the colorimetric method of Archibald (15). Urea was determined either by the manometric method of Krebs and Henseleit (1) or the colorimetric method of Archibald with isonitrosopropiophenone (16). The measurement of radioactivity was carried out according to Reid (17) with a thin mica window counter. Preparations of C^{14}-containing samples for counting were collected and dried on thin aluminum cups of known area. For orientation purposes some preparations were counted directly as dry films after adsorption of small samples on a layer of lens paper filling the bottom of aluminum cups. With care, reproducible results are obtained with this technique.

Results

Preliminary small scale experiments were carried out in which total $C^{14}O_2$ fixation was estimated for the step ornithine → citrulline (Table I). It will be noted that in all three experiments the elimination of either glutamic acid or ornithine results in a marked decrease in CO_2 fixation of the order of one-tenth to one-twelfth that of the complete system.

A large scale experiment was carried out under the conditions described in Table II. The reaction was stopped by the addition of 5.0 ml. of 1 N HCl and deproteinized by heating. The CO_2 liberated on the addition of

TABLE I

Preliminary Experiments for Estimation of $C^{14}O_2$ Fixation

Experiment	Incubation mixture	$C^{14}O_2$ fixed in protein-free medium[*]
A	Complete system	100
"	Without ornithine	10
"	" glutamic acid	3
"	" " " and NH₃	9
B	Complete system	100
"	Without ornithine	8
"	" " fumaric acid replacing glutamic acid	10
C	Complete system	100
"	Without ornithine	12
"	" glutamic acid	10

Final substrate concentrations were as follows: 3.8×10^{-2} M L-glutamate, 2.5×10^{-3} M L-ornithine, 5×10^{-3} M NH₄Cl, 1.25×10^{-2} M phosphate buffer, pH 7.15, 3.3×10^{-3} M MgSO₄, 1.5×10^{-3} M ATP, 6×10^{-3} M NaHC¹⁴O₃, and potassium ions to bring the medium to isotonicity and a total volume of 4.0 ml. Each cup contained 3.5 mg. of washed residue N. Incubation time 40 minutes at 38°.

[*] The radioactivity of the protein-free medium is expressed as relative values. 100 is equivalent to a total fixation of about 25 to 30 per cent of the C^{14} added.

acid was collected in alkali. Aliquots of the alkali samples containing the $C^{14}O_2$ were analyzed for total carbonate content by the standard Warburg manometric technique. The residual $C^{14}O_2$ was then precipitated as BaCO₃ for determination of radioactivity. Any residual $C^{14}O_2$ present in the reaction mixture was washed out by flushing with non-isotopic CO_2, which was then removed by prolonged flushing with CO_2-free air. The precipitated protein was centrifuged off, the supernatant filtered through retentive paper, and aliquots of the filtrate analyzed for citrulline and urea. 48 micromoles of citrulline were found and no urea was detected. Evapo-

ration of an aliquot to a dry film and assay for radioactivity indicated that a high CO_2 fixation had occurred.[1]

The reaction mixture after incubation and deproteinization was divided into three fractions which were diluted with non-isotopic L-citrulline. The dilutions were 5, 50, and 150 times the original citrulline present. These samples were now treated with $Ba(OH)_2$ and ethyl alcohol, according to Jones and Moeller (18), in order to remove dibasic amino acids. The precipitates were collected and aliquots assayed for radioactivity. All showed some activity due in part to the presence of some adsorbed citrulline on the crude barium salts. The precipitates were then resuspended in H_2SO_4. The $BaSO_4$ which was separated by centrifugation contained no appreciable radioactivity. Treatment of the supernatant with non-isotopic L-citrulline

TABLE II

Radioactivity of Carbon Dioxide, Citrulline, and Urea

Compound	Specific activity, counts per min. per micromole compound
Carbon dioxide..................................	420
Citrulline.......................................	413
Urea...	410

The reaction mixture for citrulline synthesis contained the following components: 3.8×10^{-2} M L-glutamate, 3.3×10^{-3} M L-ornithine, 6.6×10^{-3} M NH_4Cl, 2×10^{-3} M ATP, 1×10^{-2} M phosphate buffer, pH 7.15, 3.3×10^{-3} M $MgSO_4$, 9×10^{-3} M $NaHC^{14}O_3$, and KCl ions to bring the medium to isotonicity (activity of C^{14} measured in a Geiger-Müller counter, 36,000 counts per minute per mg. of carbon). Washed residue 28 mg. of N. The total volume of the reaction mixture was 30 ml. The mixture was incubated at 38° for 40 minutes in a closed vessel and in the presence of air.

and further precipitation with $Ba(OH)_2$ and alcohol resulted in a drop in radioactivity of the precipitate. As the activity of these precipitates was very low, isolation and identification of this fraction were not undertaken in the present studies. For precipitation of the citrulline the method of Vickery and Gordon (19) employed for other amino acids was used. The supernatant from the first $Ba(OH)_2$ treatment was freed of alcohol by evaporation and brought to pH 7.0 by the addition of H_2SO_4. After removal of $BaSO_4$, the solution was treated with $HgCl_2$ to twice the molarity of the citrulline present, and then $Ba(OH)_2$ was added to raise the pH to 9.3. The citrulline-Hg-Ba complex was separated by centrifugation, washed several times with water, alcohol, and ether, and then assayed for

[1] It has been observed that the metabolic CO_2 production under these conditions will account for a 5 per cent maximum dilution of the $C^{14}O_2$ added.

radioactivity. The analytical values obtained with all three dilutions were consistent within 5 per cent. The samples were then taken up in dilute H_2SO_4, decomposed with H_2S, centrifuged, washed, and the supernatant and washings filtered through retentive paper. The filtrate was aerated and analyzed for citrulline. The samples were concentrated *in vacuo* to a small volume, and aliquots were converted enzymatically to urea according to Cohen and Hayano (10) except that aspartic acid was used instead of glutamic acid. Urea was separated either as xanthydrol urea according to the method of Allen and Luck (20) or decomposed with urease and the CO_2 precipitated as $BaCO_3$ in the usual way.

As can be seen from Table II the specific activity per micromole of urea and citrulline is practically the same as that of the bicarbonate of the medium.

Another procedure[2] employed in a large scale experiment for estimating the incorporation of $C^{14}O_2$ into citrulline involved the quantitative adsorption of citrulline from the deproteinized reaction mixture by Zeo-Karb according to the method reported by Archibald (15). Measurements of citrulline and radioactivity indicated quantitative adsorption of both by Zeo-Karb. Approximately 80 per cent of the citrulline was eluted from the Zeo-Karb by treatment with 30 per cent H_2SO_4. Analysis of the eluate after removal of sulfate ions with $Ba(OH)_2$ revealed the same ratio of radioactivity to citrulline content as before adsorption. The eluted citrulline solution was then heated at 105° for 1 hour with concentrated sodium hydroxide to decompose the terminal ureide group to carbon dioxide which was collected as $BaCO_3$. Measurement of radioactivity of this carbon dioxide showed a specific activity per mg. of carbon which was 98 per cent that of the original bicarbonate in the medium. Of interest was the finding that washing Zeo-Karb with 30 per cent H_2SO_4 gives rise to a substance in the washings which when heated with alkali reacts with diacetyl monoxime, thus interfering slightly with the determination of citrulline.

DISCUSSION

The synthesis of citrulline and urea with practically the same specific activity as that of the $C^{14}O_2$ present originally in the medium strongly supports the position of citrulline as an obligatory intermediate in the urea cycle. The relatively low rate of $C^{14}O_2$ fixation in the absence of ornithine or glutamic acid indicates that in this system citrulline synthesis is by far the most active CO_2 fixation reaction.

Attempts to demonstrate the formation of an intermediate carbamyl-glutamic acid derivative (21) in this study were unsuccessful owing chiefly

[2] This part of the study was carried out with the collaboration of Dr. M. Hayano.

to the instability and rapid conversion of this compound. Experiments in this direction are now in progress.

The authors are indebted to Dr. Robert H. Burris, Department of Biochemistry, for samples of $C^{14}O_2$ and for facilities for estimation of radioactivity.

SUMMARY

1. The synthesis of citrulline from ornithine in the presence of $C^{14}O_2$ has been studied in washed rat liver residue. The specific activity of the isolated citrulline is of the same order of magnitude as that of the $C^{14}O_2$ added.

2. The conversion of citrulline containing C^{14} in the carbonyl position to urea by liver homogenates results in the formation of urea with the same specific activity as that of the added citrulline.

3. The significance of these findings on the intermediary rôle of citrulline in the Krebs-Henseleit cycle is discussed.

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PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE BY COUNTER-CURRENT DISTRIBUTION*

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The isolation in the pure state of the coenzyme diphosphopyridine nucleotide (DPN) has constituted a difficult problem in chemical fractionation for a number of years. At the present time, it is possible by means of relatively simple procedures to obtain crude preparations containing 40 to 60 per cent DPN from yeast in good yield (1–3). Further purification of these crude preparations (4–7) usually involves precipitation of DPN with cuprous chloride, subsequent removal of acid impurities as insoluble salts of heavy metals, adsorption of DPN on columns of Al_2O_3, and finally fractional precipitation with alcohol. The procedure is tedious and is not easily reproducible with respect to the purity or yield of the final product.

The formidable nature of this method of purification has, in fact, led to the wide-spread use of crude DPN in enzyme studies and related investigations. Although in most instances the known specificity of the DPN-linked dehydrogenases leaves little doubt that DPN itself takes part in the enzyme reactions, the question continually arises as to whether the impurities, of which the chemical nature is largely unknown, include interfering compounds. It is obvious that a reproducible and simple method for obtaining pure DPN in good yield would be desirable.

In the present report a new method, based on the counter-current distribution principle developed by Craig (8), is described for the fractionation of crude preparations of DPN (purity approximately 60 per cent). Recent applications of this technique of fractionation to other complex mixtures (9–12) have amply demonstrated that it is a powerful tool in the separation and characterization of organic compounds. By utilizing a two-phase system consisting principally of phenol and water, it has been possible with relatively few transfers to isolate DPN of high purity (at least 96 to 98 per cent pure) in yields of 70 to 80 per cent.

EXPERIMENTAL

Since the procedure of counter-current distribution depends upon the use of a two-phase system, some difficulty would be anticipated in attempts to apply the method to compounds characterized by high solubility in water and low solubility in the common organic solvents.   DPN is, of course, an excellent examp e of this type of compound.   Even when distributed in systems that contained large amounts of water in the organic solvent phase, such as 1-butanol-water or 2-butanol-water, over 99 per cent of the DPN was found in the aqueous phase ($K < 0.01$).   Phenol and certain of its derivatives (*e.g. m*-cresol), however, constituted a group of organic solvents allowing for more favorable conditions for the counter-current distribution of DPN.   In the system water-phenol at 6°, for example, approximately 90 per cent of the DPN was found in the organic phase ($K = 0.12$).   A preliminary counter-current distribution of fifteen transfers was made at 6° with 50 mg. of crude DPN with the system 2 volumes of water-1 volume of phenol.   The results of this distribution showed that most of the impurities in the preparation possessed a much higher distribution coefficient than did DPN itself.   Furthermore, DPN did not undergo an appreciable degree of transformation during the procedure.   With these data at hand, it was possible to make a more detailed analysis of crude preparations of DPN by means of the counter-current distribution technique.

Materials and Methods—The crude DPN fractionated in the present experiments consisted of several different lots of cozymase obtained from the Schwarz Laboratories[1] and containing approximately 60 per cent DPN.

Reagent grade crystalline phenol (Merck) was redistilled under reduced pressure and stored in the liquid form by the addition of 10 per cent water. All other solvents used were also redistilled in glass.

The counter-current distribution machine available for the present experiments was equipped with a glass plate at each end,[2] making it possible to observe directly the separation of the liquid phases.   This improvement was particularly useful in the purification of DPN because the water-phenol system separated rather slowly and had a tendency to emulsify in the presence of some of the impurities in cozymase.   The settling of most systems is indicated simply by transmission of light through the tubes. In the case of water-phenol, however, the layers were frequently cloudy, and a determination of separation was made by tilting the machine to the horizontal position for observation of the solvent interface by reflected light.

The DPN content of the cozymase and purified preparations obtained by

distribution was estimated according to the method of Warburg and Christian (13) as modified at the Schwarz Laboratories.[3]   A 3 to 5 mg. sample (weighed to 0.01 mg.) was dissolved in 2.00 ml. of a freshly prepared solution containing 0.20 per cent $Na_2S_2O_4$ and 1.0 per cent $NaHCO_3$.   The mixture was placed in a boiling water bath for exactly 1 minute, immediately chilled in an ice bath, and diluted to an appropriate volume with a buffer containing 1.0 per cent $NaHCO_3$ and 1.0 per cent Na_2CO_3.   The solution was oxygenated for 5 minutes and its optical density measured at 340 mμ in the Beckman spectrophotometer.   This procedure was found to be superior to the usual method of reducing DPN over a period of several hours at room temperature (13) in that it gave more reproducible extinction coefficients.

The extinction coefficient at 340 mμ afforded a precise measurement of the relative increase in purity and the yield of DPN obtained in the fractionation procedure.   Although the extinction coefficient of pure DPN reduced in solution with hydrosulfite has not been definitely established, for purposes of convenience a value of 8.5 sq. cm. per mg., according to LePage (3), was employed to estimate the DPN content of the cozymase and the samples of purified DPN.

Analysis of Crude DPN by Means of Counter-Current Distribution—Fig. 1 shows the results of a twenty-four transfer distribution of 202 mg. of cozymase.   The components of the system for distribution were equilibrated in the following proportions before the experiment: 200 ml. of water, 100 mg. of KCl, 90 ml. of phenol, and 10 ml. of ether.   Each tube of the machine contained 12 ml. of the upper (aqueous) layer and 7.8 ml. of the lower (phenol-ether) layer.   The pH of the aqueous layer was approximately 5.   The distribution was carried out in a cold room at 6° in order to minimize hydrolysis of DPN.

Potassium chloride was added because it had been previously noted that the addition of a small quantity of a strong electrolyte to the water-phenol system prevented to a considerable extent the formation of emulsions.   It was necessary to use an electrolyte which would not be an objectionable contaminant in preparations of purified DPN and which was soluble in ethanol, since the subsequent isolation of DPN involved precipitation from aqueous solution with ethanol.   KCl satisfied these requirements reasonably well.   Ether was added to raise the distribution coefficient of DPN in the water-phenol system.   As can be seen in Fig. 1, the addition of ether gave a higher distribution coefficient ($K = 0.59$) which was more favorable for fractionation.

Upon completion of the distribution, the mixture in each tube of the machine was transferred to a glass-stoppered test-tube and extracted three

[3] Gutcho, S., and Stewart, E. D., unpublished work.

times with 15 ml. of ether. The initial ether extraction resulted in the transfer to the aqueous phase of the material dissolved in the phenol and in the removal of the phenol from the system. Two additional extractions were carried out in order to insure complete removal of phenol. There . remained twenty-five aqueous solutions containing the components of the
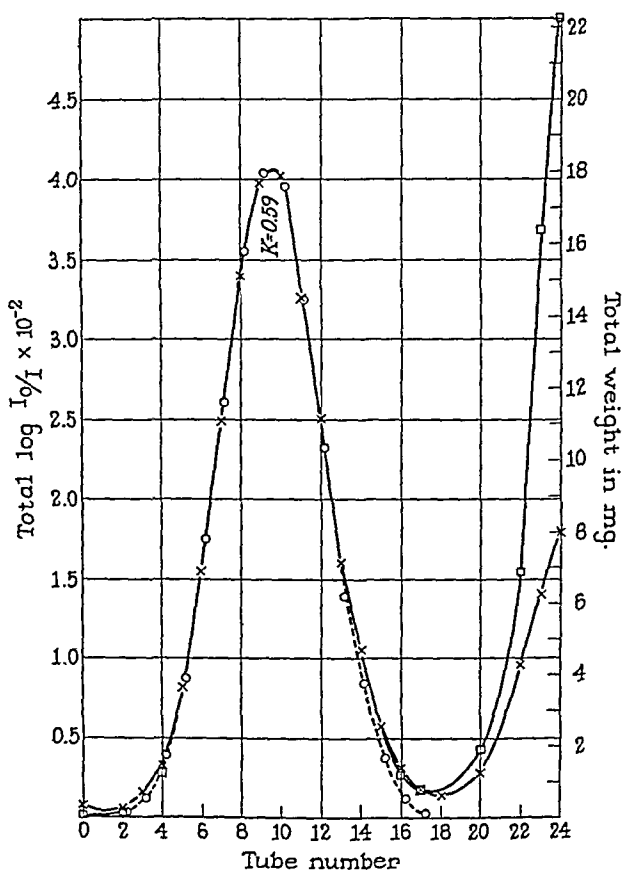

FIG. 1. Counter-current distribution of 202 mg. of cozymase in the system water-phenol-ether. ×, total optical density at 260 mμ; ○, theoretical distribution of a single substance possessing a distribution coefficient of 0.59; □, total weight in mg. after evaporation to dryness.

cozymase and the KCl. These solutions were analyzed by determining their optical density at 260 mμ and the results plotted as shown in Fig. 1. The central band, which represented DPN, coincided very closely with the theoretical distribution of a single substance possessing a distribution coefficient of 0.59. There was, however, a slight deviation beginning at Tube

13 and extending to Tube 17, indicating the presence of another substance capable of absorbing light at 260 mμ. On the basis of absorption measurements, practically all of the remaining impurities in the cozymase were in Tubes 18 to 24. It should be mentioned that the impurities in Tubes 23 and 24 were troublesome during the distribution because they tended to cause emulsification of the system, making it necessary to wait 6 to 8 minutes between transfers.

Since by the analytical method only substances absorbing light at 260 mμ could be detected, additional data were necessary before the central band could be satisfactorily interpreted. Accordingly, a number of samples from tubes on both sides of the band were evaporated to dryness and the weight of the residue determined. After correction for the amount of KCl present, the weights were plotted in Fig. 1 on a scale approximating the weight of material in the tubes of the central band. The latter values were estimated from the extinction coefficient at 260 mμ of the DPN isolated from Tubes 6 to 12, inclusive. A consideration of the amount of material present in Tubes 2, 4, 16, and 17 led to the conclusion that the central band, on a weight basis, could have been no broader than the band determined by absorption measurements and shown in Fig. 1. This finding confirmed the results of a preliminary experiment involving fifteen transfers, in which it was found that the total weight of material present in each tube of the band representing DPN corresponded exactly to the optical density at 260 mμ. It was concluded, therefore, that Tubes 4 to 12 of the central band contained, except for KCl, either a single substance or a mixture of substances possessing identical distribution coefficients in the system.

Tubes 18 to 24 contained impurities amounting to 27 per cent of the weight of the original cozymase. The absorption curve of the material in these tubes showed a pronounced maximum at 260 mμ, typical of a compound or compounds containing adenine. On reduction with hydrosulfite, however, the absorption of these impurities did not increase at 340 mμ, a finding that showed the absence of compounds containing quaternary pyridinium nitrogen. The large deviation between the weights and the absorption measurements in Tubes 22 to 24 of Fig. 1 demonstrated the presence of impurities other than those absorbing at 260 mμ.

Although very little residue remained after evaporation to dryness of the aqueous solutions obtained from Tubes 0 and 2 (Fig. 1), it should be mentioned that a yellow precipitate formed at the interface on ether extraction of the contents of Tubes 0, 1, and 2. This precipitate, which represented an impurity possessing a low distribution coefficient, was lost during the ether extraction, and its weight could not be estimated.

Isolation of DPN—Immediately after completion of the absorption analysis, the aqueous solutions obtained from Tubes 6 to 12 were combined,

filtered with suction through an inverted filter, frozen in a dry ice-acetone mixture, and lyophilized. The resulting solid material was dissolved in 4 ml. of water, and a white flocculent precipitate was obtained on addition of 40 ml. of cold absolute ethanol. The precipitate was recovered by centrifugation, washed with absolute ethanol and ether, and dried over P_2O_5 *in vacuo*. A white solid weighing 92 mg. was obtained. In an attempt to decrease the possibility of contamination with KCl, the nucleotide was redissolved in 3 ml. of water and reprecipitated with ethanol. The second precipitation presented some difficulties, however, because the compound formed an extremely fine precipitate consisting of particles of fairly uniform size (diameter about 0.4 μ) that did not coalesce over a period of 18 hours at 0°. When recovered by centrifuging at 2400 $\times$ g for 1 hour and dried over P_2O_5 *in vacuo*, the reprecipitated material weighed 82 mg. After reduction with hydrosulfite, its extinction coefficient at 340 mμ was 8.13 sq. cm. per mg., indicating a purity of 96 per cent. The starting material contained approximately 62 per cent DPN ($E_{340} = 5.3$ sq. cm. per mg.).

Fractionation of Cozymase in System, Water-Phenol-Chloroform—The experiment shown in Fig. 1 demonstrated that most of the impurities in the cozymase possessed a much higher distribution coefficient than did DPN in the system, water-phenol-ether, and could therefore be effectively removed by a few transfers. It was evident, however, that twenty-four transfers were required to eliminate impurities possessing a low distribution coefficient. That such impurities were likely to occur in crude preparations of DPN was apparent from the detection of solid material in Tubes 0, 1, and 2, which appeared on extraction with ether but was lost on removal of the ether. It was therefore desirable to devise a system in which the distribution coefficient of DPN was approximately 1. By this means it would be possible to separate with relatively few transfers impurities possessing both high and low distribution coefficients. The addition of more than 10 per cent ether to the phenol layer of the water-phenol system, in order to obtain a distribution coefficient higher than 0.59, was not feasible, however, because the density of the phenol-ether phase then approached too closely that of the aqueous phase, and the system did not separate readily. Preliminary experiments in which increasing amounts of chloroform were added to the water-phenol system showed that a distribution coefficient of approximately 1 for DPN could be obtained when the three components were used in the following proportions: 15 ml. of water, 7 ml. of phenol, and 3 ml. of chloroform. When KCl was added to the aqueous phase at a concentration of 0.10 mg. per ml. of water, the system separated very rapidly.

A twenty-four transfer distribution of 600 mg. of crude DPN was then carried out in the water-phenol-chloroform system, each tube of the

machine containing 12 ml. of the aqueous layer and 7.8 ml. of the organic solvent layer. The two phases were obtained by equilibrating before the experiment the components of the system in the following proportions: 200 ml. of water, 20 mg. of KCl, 70 ml. of phenol, and 30 ml. of chloroform. The preparation of DPN available for this experiment had been partially purified by a previous fifteen transfer distribution in the system, water-phenol-ether. The extinction coefficient of the starting material at 340 mμ after reduction with hydrosulfite was 7.4 sq. cm. per mg. (indicated purity, 87 per cent). A considerably greater amount of starting material (600 mg.) than in the previous experiment (Fig. 1) was used in order to test the feasibility of a larger scale procedure for the isolation of DPN. The experiment was carried out at 6°.

After twenty-four transfers, the contents of each tube were withdrawn from the machine, the phenol and chloroform removed by three extractions with 15 ml. of ether, and the resulting twenty-five aqueous solutions analyzed by a determination of optical density at 260 mμ. The results of this analysis are given in Fig. 2. The main band (Tubes 7 to 20), which represented DPN, was not the symmetrical type usually obtained in the counter-current distribution of a single substance but showed a precipitous rise from Tubes 7 to 10 and a more gradual decline from Tubes 12 to 19. Previous experience has shown that this type of skewed curve does not indicate the presence of impurities but is the result of a non-linear partition isotherm; *i.e.*, a shift in distribution coefficient with concentration. In view of the symmetry of the main band in the experiment shown in Fig. 1, when a relatively small amount of starting material was used (202 mg.), it was likely that the asymmetry of the band in Fig. 2 resulted from the high initial DPN concentration. A method has not as yet been devised for the calculation of theoretical curves for substances with non-linear partition isotherms. On the other hand, it has been found that the total width of the base of a skewed curve obtained with a pure substance is very nearly the same as that of the symmetrical theoretical curve. On inspection of the two curves in Fig. 2, the only detectable impurity in the main band on the basis of absorption measurements at 260 mμ occurred in Tubes 16 to 20. This impurity corresponded to that in Tubes 13 to 17 of Fig. 1. A small amount of impurity absorbing at 260 mμ was present in Tubes 21 to 24 (Fig. 2).

In order to compare the purity of the DPN on both sides of the band shown in Fig. 2, the material in Tubes 8 to 12 and in Tubes 13 to 18 was pooled separately, frozen, lyophilized, redissolved in 5 ml. of water, and precipitated by the addition of 10 volumes of absolute alcohol. Each precipitate was washed with ether and dried in a vacuum desiccator over P₂O₅. 220 mg. of DPN were isolated from Tubes 8 to 12, the extinction

coefficient at 340 mμ after reduction with hydrosulfite being 8.36 sq. cm. per mg. (indicated purity, 98 per cent). Tubes 13 to 18 yielded 209 mg. of DPN, of which the extinction coefficient at 340 mμ after reduction was 8.26 sq. cm. per mg. (indicated purity, 97 per cent). Both preparations were amorphous but perfectly white in color. The slightly lower extinction coefficient of the DPN from Tubes 13 to 18 was probably due to
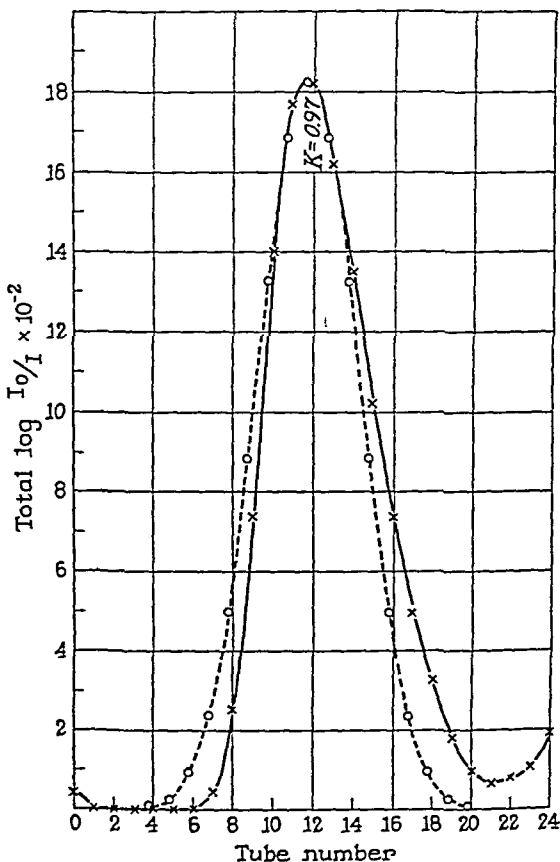

Fig. 2. Counter-current distribution of 600 mg. of partially purified DPN in the system water-phenol-chloroform. ×, total optical density at 260 mμ; O, theoretical distribution of a single substance possessing a distribution coefficient of 0.97.

small amount of impurity in Tubes 16 to 18. Reprecipitation of the DPN from Tubes 13 to 18 did not produce any change in its extinction coefficient. On the basis of extinction coefficients at 340 mμ, a total of 80 per cent of the DPN in the starting material was recovered.

Procedure for Isolation of DPN on Large Scale—It was apparent from the data presented above that the use of an initial DPN concentration of much

more than 3 per cent in the water-phenol-chloroform system would result in an excessive shift in the distribution coefficient. In larger scale procedures, it would therefore be necessary to increase the volume of the solvents above the capacity of the present counter-current distribution machine. The data also indicated, however, that the number of transfers required to effect satisfactory purification was sufficiently small to render the fractionation method feasible without resorting to the machine. Accordingly, the following experiment was carried out in order to test the practicability of a larger scale procedure for the isolation of DPN. Although only 1 gm. of cozymase was available for fractionation, it was evident that larger amounts could have been used if appropriate increases had been made in the volume of the solvents.

The first step in the purification was a four transfer distribution at 6° of 995 mg. of cozymase in the system, 12 ml. of water-12 ml. of phenol. The water phase contained 0.10 mg. of KCl per ml. Heavy glass-stoppered test-tubes were used for the distribution. After each equilibration, the tubes were centrifuged because of the tendency for the impurities possessing a high distribution coefficient to cause emulsification. The upper layers were transferred from one tube to the next by a vacuum-operated siphon. Tubes 0, 1, and 2 contained practically all the DPN because of its low distribution coefficient ($K = 0.12$) in the water-phenol system. Tubes 3 and 4 contained the bulk of the impurities of high distribution coefficient, corresponding to the material in Tubes 18 to 24 of the distribution shown in Fig. 1. The contents of each tube were then extracted three times with 15 ml. of ether. The resulting aqueous solutions obtained from Tubes 0, 1, and 2 were combined, frozen, and lyophilized. The residue was dissolved in 10 ml. of cold water, precipitated by the addition of 100 ml. of cold absolute alcohol, recovered by centrifugation, washed with ether, and dried *in vacuo* over P_2O_5. This preparation of partially purified DPN consisted of yellow resinous material and weighed 684 mg. After reduction with hydrosulfite its extinction coefficient at 340 mμ was 7.14 sq. cm. per mg. Since the extinction coefficient of the original cozymase was 5.25 sq. cm. per mg., the yield in terms of DPN recovered was 92 per cent. The purity of the DPN had been increased from 62 to 84 per cent.

The aqueous solutions from Tubes 3 and 4 were evaporated to dryness at 100°. Tube 3 contained 76 mg. and Tube 4, 144 mg. of impurity.

The second step in the isolation procedure consisted in distributing in the water-phenol-chloroform system the partially purified DPN obtained from Tubes 0, 1, and 2. This distribution was carried out under conditions similar to those of the experiment shown in Fig. 2. The number of transfers, however, was reduced to sixteen. 453 mg. of DPN were collected from Tubes 4 to 11, the extinction coefficient at 340 mμ being 8.25 sq. cm. per mg. after reduction with hydrosulfite. The indicated purity of the

compound was therefore 97 per cent. The over-all yield of DPN obtained by the two distributions of 995 mg. of cozymase was 72 per cent.

Chemical Analysis of Purified DPN—Table I shows the results of elementary analysis of samples of DPN isolated from the distributions shown in Figs. 1 and 2. Both samples had been stored in a desiccator over P_2O_5 before analysis. Residual water was determined by heating the samples to constant weight at 100° *in vacuo*. In order to estimate the extent of contamination by KCl, chlorine analyses were carried out in addition to analyses for C, H, N, and P. It can be seen from the data in Table I that both samples gave low C, N, and P and high H values. Sample 2, which was purer than Sample 1 on the basis of extinction coefficients at 340 mμ ($E = 8.36$ *versus* 8.13 sq. cm. per mg.), gave C:N and N:P ratios in exact agreement with the theory, whereas the C:N ratio for Sample 1 was

TABLE I

Analysis (Per Cent) of Samples of Purified DPN*

Sample	Residual water	C	H	N	P	Cl	C:N	N:P
Calculated for $C_{21}H_{27}N_7P_2O_{14}$ (mol. wt., 663.5)		38.01	4.10	14.78	9.35		2.572	1.58
Found, Sample 1†	5 46	37.75	4.44	14.38	9.08	Trace	2.62	1.58
" " 2‡	5.30	37.33	4.40	14.52	9.18	0.10	2.571	1.58

* Analyses were performed by Mr. D. Rigakos and Miss Theta Spoor of The Rockefeller Institute for Medical Research and by Dr. A. Elek, 4763 West Adams Boulevard, Los Angeles 16, California.

† DPN isolated from Tubes 6 to 12 of the distribution shown in Fig. 1.

‡ DPN isolated from Tubes 8 to 12 of the distribution shown in Fig. 2.

somewhat high. In general, the analytical values indicated that an appreciable amount of residual water was present in the samples even after they were dried at 100° *in vacuo*. Attempts to attain a completely anhydrous state were unsuccessful, however, because DPN decomposed when heated at temperatures higher than 100°.

Activity of Purified DPN As Coenzyme—As a final check on the extent of purification of DPN, it was desirable to compare the activity of the purified DPN as a coenzyme with that of a crude cozymase preparation. The lactic acid dehydrogenase system of rat liver was found to offer a convenient method for estimating DPN by enzyme assay. This determination was based on the finding that the DPN-cytochrome c reductase activity of rat liver homogenates was much higher than the lactic acid dehydrogenase activity.[4] When sufficient cyanide was added to inhibit cytochrome

[4] Hogeboom, G. H., unpublished work.

oxidase, a determination of lactic acid dehydrogenase activity could be made by following spectrophotometrically at 550 mμ the rate of reduction of cytochrome c. Under the conditions noted below the reaction proceeded linearly with time for approximately 10 minutes, and the rate was proportional to the DPN concentration until relatively large amounts of DPN had been added.

Fig. 3 shows a comparison of the coenzyme activity in the lactic acid dehydrogenase system of a cozymase preparation (E_{340} = 5.3 sq. cm. per mg., indicated purity 62 per cent) with that of purified DPN (E = 8.36
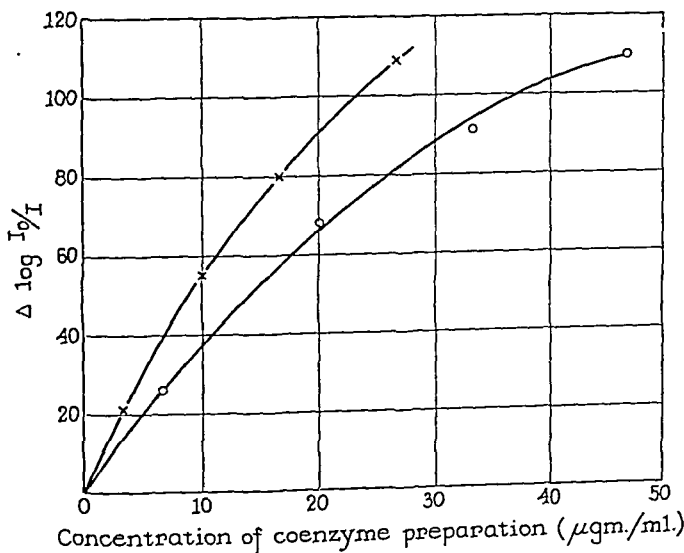

FIG. 3. Comparative activity of crude cozymase and purified DPN as hydrogen carriers in the lactic acid dehydrogenase system of rat liver. $\times$, increase in optical density at 550 mμ over a 5 minute period on the addition of increasing amounts of purified DPN; $\bigcirc$, increase in optical density at 550 mμ over a 5 minute period on the addition of increasing amounts of crude cozymase.

sq. cm. per mg., indicated purity 98 per cent). The reaction mixtures were made up by adding the following components in order: 0.20 ml. of 0.50 M K$_2$HPO$_4$-KH$_2$PO$_4$ buffer, pH 7.4; 0.20 ml. of 0.82 M nicotinamide; water to give a final volume of 3.00 ml.; 0.30 ml. of liver extract, diluted 1:50 with 0.88 M sucrose; 0.30 ml. of 10^{-3} M NaCN; 0.40 ml. of 2.2 $\times$ 10^{-4} M oxidized cytochrome c; varying amounts of DPN or cozymase dissolved in 0.05 M potassium phosphate buffer, pH 7.4; and 0.20 ml. of 0.11 M sodium lactate. The control reaction mixture contained all components except DPN.

The liver extract was prepared as described previously (14) by homogenizing 1 gm. of rat liver in 9 ml. of 0.88 M sucrose, and centrifuging the

homogenate for 20 minutes at $600 \times g$ in order to remove residual intact cells and free nuclei. The cell-free supernatant was used as a source of lactic acid dehydrogenase and DPN-cytochrome c reductase. Cytochrome c was maintained in the oxidized form by the addition of HCl to a final concentration of 0.01 M. The experiment was carried out at 22°.

After the addition of sodium lactate, the reaction mixtures were quickly transferred to cuvettes and the increase in the optical density at 550 mμ followed at intervals of a minute in the Beckman spectrophotometer. In Fig. 3 the increase in absorption over a 5 minute interval is plotted against the concentration of DPN or cozymase. It can be seen that at each level of enzyme activity the approximate ratio of crude cozymase to purified DPN yielding an equivalent reaction rate was 1:0.63. The results of this experiment are in agreement with the DPN content of the two preparations, as calculated from the extinction coefficients at 340 mμ after reduction with hydrosulfite. The experiment also demonstrated that the DPN isolated by the counter-current distribution method is active as a coenzyme.

<center>DISCUSSION</center>

Purity of DPN Isolated by Counter-Current Distribution—On the basis of several criteria of homogeneity, it is probable that the DPN obtained by the counter-current distribution of crude preparations of cozymase contained, aside from residual moisture, no more than a few per cent of impurities. Although not an entirely reliable measure of purity, the extinction coefficient of the DPN at 340 mμ after reduction with hydrosulfite closely approached maximum values expected for pure DPN reduced under similar conditions. In this respect, Drabkin (15) has shown that the occurrence of either transformation or reoxidation during the reduction of DPN in solution results in significantly lower extinction coefficients than corresponding values obtained for reduced diphosphopyridine nucleotide (DPNH$_2$) isolated by Ohlmeyer's procedure (16). The extinction coefficient at 340 mμ for pure anhydrous DPNH$_2$ is 9.43 sq. cm. per mg. (16). On the basis of Drabkin's data, the value for pure anhydrous DPN, reduced in solution with hydrosulfite, was estimated to be approximately 8.8 sq. cm. per mg. The extinction coefficients of the preparations of DPN isolated by counter-current distribution, after correction for residual water content, were 8.63 to 8.83 sq. cm. per mg.

Elementary analysis of the purified DPN was rendered somewhat difficult by the fact that the compound was hygroscopic and contained residual moisture after having been stored over P$_2$O$_5$ for prolonged periods in a vacuum desiccator. A completely anhydrous state was apparently not attained even after heating the compound *in vacuo* at 100°. In general, it

may be stated, however, that the analytical figures compared favorably with similar values reported in the literature for DPN considered to be pure (6, 7, 13, 17) and indicated that preparations containing at least 96 to 98 per cent DPN can be readily isolated by means of counter-current distribution.

Finally, it should be pointed out that the curves representing DPN (Figs. 1 and 2) obtained from the counter-current distribution of cozymase were in close agreement with the theoretical distribution of a single substance. It has been shown that agreement between experimental and calculated curves is an important criterion of homogeneity (9, 10, 12).

Remarks on Fractionation and Isolation Procedure—Counter-current distribution often, as in the case of the penicillins (9, 10), makes possible the separation and isolation of unstable compounds with a minimum of decomposition. In the present experiments with DPN, a substance that is easily hydrolyzed at both acid and alkaline pH, the temperature was maintained at 6° or below, and the pH of the aqueous phase of the system was approximately 5. In spite of the mildness of these conditions, however, a consideration of the curves shown in Figs. 1 and 2 brought up the possibility that a slight amount of transformation of DPN may actually have occurred. Thus the results of the distribution shown in Fig. 1 indicated that fragments of the DPN molecule containing adenine possessed a very high distribution coefficient and should have appeared almost exclusively in Tubes 23 and 24 of the experiment shown in Fig. 2. If a continuous, slow hydrolysis occurred during the distribution, however, these fragments would not be sharply localized in Tubes 23 and 24 but would extend from the right side of the DPN band to Tube 24. The fact that the total optical density of the material in Tubes 20 to 23 of Fig. 2 did not approach zero but remained almost constant could be accounted for on this basis.

Previous investigations (5) have shown that the weakest point of the DPN molecule occurs at the linkage between ribose and pyridine nitrogen and that the initial step in the hydrolysis of DPN yields free nicotinamide. In the method of isolation of DPN used in the present experiments, involving precipitation from aqueous solution with ethanol, free nicotinamide would be eliminated from the final preparation because of its solubility in ethanol. Hydrolytic products from the remainder of the molecule might be precipitated by ethanol and thus could occur as possible contaminants.

It should also be pointed out that heavy metals, which might possibly be present in small amounts in crude DPN, would remain in the aqueous phase of the water-phenol system and therefore would not contaminate DPN purified by counter-current distribution.

A comparison of the distribution coefficient of DPN and triphospho-

pyridine nucleotide (TPN) and an investigation of the behavior of the latter substance on counter-current distribution would be of considerable interest. TPN was not available in sufficient amounts, however, to permit such a study.

The authors are indebted to Dr. Lyman C. Craig for his interest and helpful suggestions.

SUMMARY

A method, based on counter-current distribution, is described for the fractionation of crude preparations of diphosphopyridine nucleotide (DPN). DPN of high purity (at least 96 to 98 per cent pure) was readily isolated in yields of 70 to 80 per cent by distribution in a two-phase system consisting principally of phenol and water. The procedure was demonstrated to be suitable for the isolation of pure DPN on a large scale.

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THE ACTION OF ETHANOLAMINE, METHYLETHANOLAMINE, AND DIMETHYLETHANOLAMINE ON LIPIDE PHOSPHORYLATION*

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The administration of a single dose of choline stimulates the formation of phospholipides in the liver (2, 3) and in the small intestine (4) of rats on low protein diets. Like choline, ethanolamine is an essential constituent of phospholipides in tissues and one may suspect that it will exert a similar action. Moreover, choline can conceivably be formed by methylation of ethanolamine through the intermediate stages of methyl- and dimethylethanolamine. There is already rather extensive evidence for the occurrence of such a process in living organisms (5–11), although, in this respect, considerable species differences were noted.[1]

In the present study the action of ethanolamine, methyl-, and dimethylethanolamine on the formation of total phospholipides in the liver and small intestine has been investigated with the aid of radioactive phosphorus as an indicator.[2] The results have been compared with those of simultaneous experiments in which an equivalent amount of choline was given. Moreover, since apparently similar effects on the total phospholipides may actually be the result of quite different actions on the individual phospholipide fractions, in a few experiments the separation of the choline-containing from the non-choline-containing phospholipides of the liver has been attempted, and the determinations have been carried out on the separated fractions.

Since the completion of our investigation, of which the main findings have been reported in summarized form (1), a paper by Platt and Porter (14)

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[1] Unlike the rat (5) and a type III pneumococcus (6), the chick (12) and one mutant strain of *Neurospora crassa* (13, 8) seem to be unable to carry out the conversion of ethanolamine into methylethanolamine.

[2] The P^{32} was supplied by the Clinton Laboratories, Oak Ridge, Tennessee, on allocation from the United States Atomic Energy Commission. Part of the methyl- and dimethylethanolamine used in the experiments was graciously offered by the Carbide and Carbon Chemical Corporation, New York.

has appeared concerning the action of ethanolamine on the rate of formation of phospholipides in the liver. On this point, their and our results are substantially in agreement.

<div align="center">EXPERIMENTAL</div>

Male albino rats (100 to 110 gm.) were maintained for 7 days on a low fat, low protein diet (Diet 26 (3)) in which a solution of pure B vitamins (15) was incorporated daily. Some of the animals received a single large dose of a commercial preparation of salad oil 18 hours before death. Three to seven animals were used in each experiment. One of them was used as a control and received 1 cc. of water by stomach tube. The others received one dose of the substances to be tested (1 cc. of a 0.2 M solution of the chlorides by stomach tube).[3] 5 minutes later the rats were injected intraperitoneally with a solution of Na_2HPO_4 (10 to 20 γ of P) containing 2 to 4 microcuries of radioactive phosphorus and after 6 hours they were killed by decapitation. The lipides were extracted from the liver and the small intestine and their radioactivity and phosphorus content determined. The analytical procedures and the method for calculating and expressing the results have been described (3).

For the separation of the choline-containing from the non-choline-containing phospholipides, the chloroform solution of the lipides extracted from one or more livers was used. After evaporation of the solvent, the residue was dissolved in methanol, and the solution filtered and treated according to Taurog et al. (16). The radioactivity, phosphorus, and often also choline (17) were determined in aliquots of the solution before and after adsorption on MgO.

<div align="center">*Results*</div>

The data on the radioactivity and specific activity of the total phospholipides in the liver and small intestine are reported in Tables I and II. In order to evaluate the statistical significance of our results, the differences observed in each experiment between the rats receiving the substances and the control receiving water have been averaged and the t test of significance (18) applied to the averages.[4] The mean increase over the controls, the value of t, and the degree of probability (P) for a chance occurrence of the increase are indicated in Tables I and II.

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[3] Ethanolamine (Eastman Kodak), methylethanolamine (Carbide and Carbon Chemical Corporation), dimethylethanolamine (Carbide and Carbon Chemical Corporation, Eastman Kodak). These substances were identified by determinations of specific gravity, boiling point, acid equivalent, ammonia evolved by alkaline periodate, and formol titration. The titer of the solutions of choline chloride (Merck and Company) was estimated by determinations of N and Cl.

[4] Substantially the same conclusions are reached if the statistical treatment is applied to the differences between the means of each experimental group.

TABLE I

*Total Radioactivity and Specific Activity of Phospholipides in Liver of Rats Receiving Water, Ethanolamine, Methylethanolamine, Dimethylethanolamine, and Choline**

Experiment No.	Total radioactivity					Specific activity				
	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline
	r.r.u.	*r.r.u.*	*r.r.u.*	*r.r.u.*	*r.r.u.*					
1†	281	428			586	86	126			133
2†	426	465		581	485	86	128		123	96
3†	291	462		803	495	81	102		147	107
	385					85				
4†	408	559	592	734		106	132	110	159	
5	307	421			394	86	115			103
6	373	496		542	500	102	126		119	104
7	300	626		729		78	135		144	
8	352	429	636			93	150	179		
		558					152			
9	534		828	748		96		179	142	
			644					164		
10	402	500	535	579	475	96	118	122	163	115
		547			433		140			121
11	439	680			513	100	141			106
12	482		575	930		85		95	148	
Averages‡	383 (398)	514 (532)	635 (643)	705 (705)	485 (463)	91 (92)	130 (134)	141 (148)	143 (143)	110 (110)
Mean increase over controls		147	183	316	119		37	46	53	19
t		6.89	4.68	7.23	4.19		8.89	3.01	9.33	4.39
P		<0.01	<0.01	<0.01	<0.01		<0.01	<0.05	<0.01	<0.01

* The radioactive values are expressed in relative radioactive units (r.r.u.), the dose of P^{32} injected into the rat being considered as 10^4 r.r.u. "Total radioactivity" is the number of r.r.u. in the lipides of the whole organ. "Specific activity" is the ratio of the total radioactivity to the mg. of lipide phosphorus in the organ of a 100 gm. rat. *t* is the test of significance as applied to the mean increase over the control of the same experiment. *P* is the degree of probability for a chance occurrence of this increase.

† These rats received 1.5 gm. of oil 18 hours before death (12 hours before receiving P^{32}).

‡ The figures in parentheses are the averages of the values obtained on the rats to which no oil was given.

It is apparent that, in the liver of the rats fed any of the substances tested, both the radioactivity and the specific activity are markedly in-

creased and that all the increases have a considerable degree of statistical significance.

On the basis of the average values, the stimulating effect on the lipide phosphorylation in the liver is increasing in the order, choline, ethanolamine, methyl-, and dimethylethanolamine.

TABLE II

Total Radioactivity and Specific Activity of Phospholipides in Small Intestine of Rats Receiving Water, Ethanolamine, Methylethanolamine, Dimethylethanolamine, and Choline

Experiment No.	Total radioactivity					Specific activity				
	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline	Water (controls)	Ethanolamine	Methyl-ethanolamine	Dimethyl-ethanolamine	Choline
	r.r.u.	*r.r.u.*	*r.r.u.*	*r.r.u.*	*r.r.u.*					
1*	125	184			229	45	59			69
2*	213	206		224	236	61	74		81	64
3*	226	179		261	181	61	57		92	58
	194					59				
4*	199	156	189	239		61	66	59	77	
5	120	124			184	52	52			60
6	161	222		214	185	55	77		68	64
7	160	206		191		55	70		70	
8	109	116	128			41	48	58		
		168					60			
9	88		171	131		51		70	70	
			124					76		
10	167	172	211	215	160	51	60	71	74	67
		167		183			63			66
11	210	198			250	65	66			76
12	216		208	257		58		61	79	
Average*	168	174	172	216	201	55	63	66	76	65
	(154)	(171)	(168)	(201)	(192)	(53)	(62)	(67)	(72)	(66)
Mean increase over controls		9	27	41	23		9	15	21	9
t		0.88	1.86	7.18	1.50		3.56	4.92	9.14	3.1
P		>0.05	>0.05	<0.01	>0.05		<0.01	<0.01	<0.01	<0.0

* See explanations for Table I.

Essentially similar effects have been observed in many, but not in all experiments on the small intestine. Accordingly, the mean increases over the controls, especially for the total radioactivity, are often small and always less marked than in the liver. However, all the differences in the specific activity values are clearly significant. In this respect, it should be

pointed out that our determinations on the small intestine have been made on the whole organ, *i.e.* on both mucosa and muscle. In the latter tissue, the rate of phospholipide turnover is lower and perhaps it is modified to a lesser degree (or not at all) by the substances given to the animals.

From the limited number of data available, it seems that the administration of oil 18 hours before death did not affect appreciably these results either in the liver or in the intestine.[5]

Table III shows the results of determinations made in order to test the reliability of the method for the separation of the choline- and non-choline-

TABLE III

Total,* Choline-Containing,† and Non-Choline-Containing‡ Phospholipides of Rat Liver before and after Adsorption on MgO

Substance given	Sample	Before adsorption			After adsorption			Choline-containing phospholipide not adsorbed	Non-choline-containing phospholipide adsorbed
		Total phospholipide	Choline-containing phospholipide	Ratio, choline-containing to total	Total phospholipide	Choline-containing phospholipide	Ratio, choline-containing to total		
		mg.	mg.		mg.	mg.		per cent	per cent
Water	A	104	51	0.49	66	48	0.73	94	66
	B	83	44	0.53	58	41	0.71	93	56
	C	109	51	0.47	61	48	0.79	94	78
Ethanolamine	D	82	41	0.50	51	39	0.76	95	61
	E	105	56	0.53	66	53	0.80	95	72
	F	84	44	0.52	49	41	0.84	93	80
Methylethanol-amine	G	92	53	0.58	59	52	0.88	98	82
	H	94	49	0.52	52	48	0.92	98	91
Dimethyl-ethanolamine	I	102	59	0.58	65	57	0.88	97	81
	J	74	41	0.55	46	42	0.91	102	86
Choline	K	84	64	0.76	58	60	1.03	94	100
	L	86	62	0.72	61	61	1.00	98	100

* Total phospholipide = mg. of lipide P $\times$ 22.7.

† Choline-containing phospholipide = mg. of choline $\times$ 6.7.

‡ Non-choline-containing phospholipide = (mg. of total phospholipide) − (mg. of choline-containing phospholipide).

containing phospholipides in our lipide extracts. On the basis of the choline values, it appears that, after treatment with MgO, practically all of the choline-containing phospholipides (93 to 100 per cent) are left in the solution. However, the amount of phosphorus which escaped adsorption is

[5] However, in four out of five experiments the intestine of the control rats which had received oil exhibited values for the total radioactivity (and, to a lesser extent, also for the specific activity) which were higher than the general averages. Similar increases in the intestinal lipides of rats on Diet 26 after the administration of a large dose of oil 6 hours before death have been noted previously (3).

often greater than that which would have been expected from the choline figures, this excess representing from 9 to 44 per cent of the non-choline-containing phospholipides assumed to be present in the original extract. All of the non-choline-containing phospholipides seem to have been adsorbed only in the two experiments on the liver of rats which received choline. In these experiments the ratio of choline to phosphorus in the solution after adsorption approximates 1, as in the determinations of Taurog *et al.* (16). These findings suggest the possibility that, when choline or choline precursors are absent from the diet, increased amounts of

TABLE IV

Total Radioactivity and Specific Activity of Rat Liver Phospholipides in Fractions Not Adsorbed and Adsorbed by MgO

Substance given	No. of analyses	Not adsorbed (choline-containing phospholipide)	Adsorbed (non-choline-containing phospholipide)	Choline-containing phospholipide (a)	Non-choline-containing phospholipide (b)	Ratio $\frac{(a)}{(b)}$
				Per cent increase over controls		
Water (controls)	4	227 ± 8 (91 ± 9)	141 ± 12 (92 ± 29)			
Ethanolamine	5	310 ± 46 (131 ± 25)	218 ± 21 (127 ± 12)	37 (47)	54 (36)	0.7 (1:3)
Methylethanolamine	2	328 ± 14 (133 ± 3)	205 ± 6 (128 ± 13)	45 (48)	45 (39)	1.0 (1.2)
Dimethylethanolamine	3	416 ± 49 (156 ± 39)	186 ± 17 (127 ± 6)	83 (77)	32 (38)	2.6 (2.0)
Choline	3	318 ± 15 (118 ± 8)	149 ± 19 (115 ± 9)	40 (31)	5 (25)	8.0 (1.2)

* The figures preceded by the ± sign indicate the standard deviations. The values in parentheses are those of the specific activity. For other explanations see Table I.

substances containing phosphorus, but not choline, may be present in the lipide extracts of the liver and, like lecithin, remain in the methanol solution after treatment with MgO. This hypothesis is now being investigated. At present, it is apparent that, at least in most of our experiments, the significance of the results obtained by the procedure which we have used is open to question. With this reservation, in Table IV we have merely recorded the average data of these determinations and their standard deviations. After the administration of ethanolamine, methyl-, and dimethylethanolamine, the radioactivity values in both phospholipide fractions were consistently higher than the corresponding values in the controls receiving

water. In the group receiving choline, the radioactivity was definitely elevated only in the choline-containing fraction.

When the changes in the radioactivity of the two fractions are compared with each other, the *absolute* increase was always higher in the choline-containing phospholipides. However, the *per cent* increase over the control value was greater in the non-choline-containing fraction after ethanolamine was given, and was approximately the same in the two fractions from the rats receiving methylethanolamine. On the other hand, the increase was proportionately higher in the non-choline-containing phospholipides after dimethylethanolamine was given, and, even more so, after choline administration.

As for the specific activities, in all experimental groups the values were higher than in the controls, in the choline-containing as well as in the non-choline-containing phospholipides. Except for the rats receiving dimethyl-ethanolamine, the average figures were of the same order of magnitude in the two fractions. However, the individual data were quite irregular, and in the various experiments of the same group the specific activity was sometimes greater in the choline-containing and sometimes in the non-choline-containing fraction.

In view of these inconsistencies, it is not easy to state definitely whether or not any of the substances which we have tested stimulated preferentially the formation of lecithin or of cephalin. Indeed, under the conditions of our experiments, it seems that the evidence for a proportionately greater formation of one phospholipide fraction would be conclusive only if the per cent increase in the total radioactivity of this fraction were greater and if the specific activity were also consistently higher or, at least, not lower than that of the other fraction. However, it must be pointed out that, because of the likely presence in the lipide extracts of variable amounts of P-containing substances other than the typical lecithin and cephalin (see above), the computation of the specific activity in the separated fractions was probably subject to an error greater than that of the radioactive determinations. On the other hand, if lecithin and cephalin were synthesized in the liver from a common immediate precursor containing P (*e.g.* glycero-phosphate or phosphatidic acid), the comparison between the radioactivity values would be a direct indication of the relative amounts of phospholipides newly formed in the two fractions.

DISCUSSION

Present findings show that in both liver and intestine of rats on a low fat, low protein diet the administration of a single dose of ethanolamine or of its products of partial methylation stimulates the formation of total phospholipides. The stimulation appears to be of the same order of magni-

tude or greater than that caused by the administration of an equivalent amount of choline.[6]

The effect of choline on lipide phosphorylation in the liver has been correlated with the action of this substance in preventing fatty liver (and hemorrhagic kidneys in weanling rats). Other lipotropic substances such as betaine (19) and methionine (20) also cause an increase in the formation of total phospholipides in the liver. Dimethylethanolamine, which in the present experiments stimulated markedly the lipide phosphorylation, was previously found to be lipotropic (7). On the other hand, ethanolamine is said to be ineffective (21, 22), although in very young rats the administration of this substance, alone or together with methionine, to a certain extent decreases the fat infiltration (23, 24) and prevents hemorrhagic kidneys (24). An even more definite lack of parallelism between stimulation of lipide phosphorylation in the liver and lipotropic activity has been noted for cysteine and cystine (20). In other words, from the present evidence, it appears that those substances which exert a marked lipotropic action also stimulate the formation of liver phospholipides, but that the reverse is not true. Apparently, the lipotropic action is more specific than the stimulation of lipide phosphorylation.

As mentioned above, the adequacy of the separation of the choline-containing and non-choline-containing phospholipides i open to question, and a further uncertainty in the interpretation of the data is introduced by the irregularity in the specific activity values as determined in the separated fractions. If it were assumed that in the conditions of our experiments the radioactivity data may be a reliable indication of the relative amounts of choline- and non-choline-containing phospholipides formed during the 6 hours of the experiments, it would appear that, after choline was given, the stimulation of lipide phosphorylation in the liver involves almost exclusively the choline-containing fraction, a finding which would be in line with previous results of Entenman *et al.* (25) on the liver of dogs receiving choline. On the other hand, after ethanolamine or its partially methylated derivatives were fed, the formation of both lecithin and cephalin is increased, but the extent of the relative increase in each fraction varies with the substance given.

As a tentative explanation of these findings, it may be pointed out that the protein level in our experimental diet was as low as 5 per cent, and that casein contains only minute amounts of glycine, a likely precursor for ethanolamine (5). In spite of the easiness with which glycine can be syn-

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[6] However, it should be pointed out that, according to our previous data (3), the choline effect on lipide phosphorylation in the liver and intestine of rats on Diet 26 is markedly enhanced by the simultaneous administration of fat.

thesized in the body, it is perhaps not unreasonable to assume that in our control rats the availability of ethanolamine, as well as that of choline, could represent a limiting factor for the synthesis of phospholipides. When large amounts of preformed ethanolamine were administered part of the substance given may have been utilized directly as a building stone for the formation of cephalin, and another part indirectly as a methyl acceptor for the synthesis of choline. One can thus understand that in these experiments an increased formation of both phospholipide fractions occurred, the synthesis of lecithin being then limited only by the amounts of available methyl groups. After methyl- or dimethylethanolamine was given, a more marked synthesis of lecithin became possible, since for each newly formed molecule of choline, only two, or one, additional methyl groups were required. When large amounts of choline are fed, it is probable that part of the choline reaching the liver is destroyed by the action of choline oxidase (26, 27) and thus is not used in the synthesis of phospholipides. On the other hand it is possible that dimethylethanolamine is introduced as such into the molecule of a phospholipide (or a phospholipide precursor) without previous methylation to choline, and by this process escapes the action of choline oxidase. This might perhaps explain the higher rate of lecithin formation after dimethylethanolamine is given than after choline administration.

As for the finding that not only ethanolamine but also its products of partial methylation caused an increase in the radioactivity of the non-choline-containing fraction, it is conceivable that in these experiments some ethanolamine originated from the compounds fed. Such a demethylation should probably occur through reactions other than those involved in transmethylation, since dimethylethanolamine apparently is not an effective methyl donor for the synthesis of methionine (7, 28). On the other hand, if the possibility of a direct introduction of partially methylated ethanolamine in the phospholipide molecule is accepted, such atypical phospholipides may be partly adsorbed on MgO and thus give higher values in the cephalin fraction. It is obvious that the speculations outlined above are merely working hypotheses and that a number of alternative explanations may be suggested.

SUMMARY

Rats maintained on a low casein, low fat diet were given by stomach tube a single dose of ethanolamine, methylethanolamine, dimethylethanolamine, or choline. The controls received water. The animals were then injected with isotopic phosphate and the radioactivity and the phosphorus content determined in the lipides of the liver and small intestine.

All the substances tested stimulated the formation of total phospholipides in both tissues. The stimulation by ethanolamine and by the products of its partial methylation was of the same order of magnitude or greater than that observed after choline was given.

In a number of experiments the liver phospholipides have been separated into choline-containing and non-choline-containing by adsorption on magnesium oxide. Under the conditions of the present experiments, the adequacy of the separation and the significance of the results obtained on the separated fractions are somewhat doubtful. To the extent of their reliability, these results showed that after ethanolamine, methyl-, and dimethylethanolamine were given, the radioactivity was increased in both phospholipide fractions, but the extent of the relative increase in each fraction varied with the substance administered. After choline was given, the increase was confined to the choline-containing phospholipides.

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THE EFFECT OF CYSTEINE, HISTIDINE, AND METHIONINE ON THE PRODUCTION OF POLYCYTHEMIA BY COBALT*

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The fact that cobalt administered daily in small amounts either orally or parenterally will produce a polycythemia is now well established. The polycythemia has been produced in a number of species of animals, including the rat, mouse, rabbit, dog, duck, and frog, and is characterized by an increase in the erythrocyte count and hemoglobin and hematocrit values without any significant alteration, either quantitatively or qualitatively, in the leucocytes (1). There is a distinct increase in the total blood volume due to an increase in the number of circulating erythrocytes, the plasma volume remaining essentially unaltered (2, 3).

The mechanism involved in the production of polycythemia by cobalt has received some attention. Previous work in this laboratory has indicated that there is some active stimulus to erythropoiesis, since a distinct reticulocytosis precedes the rise in the erythrocyte count (4). Barron and Barron (5) have suggested that cobalt may inhibit cellular respiration and thus produce a compensatory polycythemia for the purpose of increasing oxygen transport to the cells. In support of this hypothesis they have reported that the administration of ascorbic acid, allegedly involved in cell respiration, inhibits the production of polycythemia by cobalt in the rabbit. Other studies in this laboratory (6) add some indirect support to such a hypothesis by demonstrating that cobalt does not alter the oxygen-carrying capacity of hemoglobin nor does it form a "methemoglobin" in the rat. Thus if cobalt produces a compensatory polycythemia by interfering with the respiratory process, it must be the internal or cellular respiration which is affected rather than the external respiratory process.

The effect of several nitrogenous compounds on the action of administered cobalt in the animal organism has been investigated. Davis (7) reported that choline administered orally to dogs will completely inhibit the pro-

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duction of polycythemia by cobalt. This observation was not confirmed in this laboratory with the rat as the experimental animal (8). Griffith and his coworkers (9) have observed that cysteine, and to a lesser extent cystine, but not methionine, will greatly decrease the toxicity of orally administered cobalt in the rat as evidenced by growth response. However, they did not study the possible effects on hematopoiesis. These investigators attributed the toxic effect of cobalt to a "fixation of sulfhydryl compounds in tissues with resulting interference with oxidative mechanisms."

Another amino acid, histidine, has been investigated in connection with the toxicity of cobalt. Burk et al. (10) have observed that histidine decreases the toxicity of cobalt in certain bacteria and increases the growth and respiration of cobalt-treated microorganisms and cultures of various animal tissues. Burk et al. (11), as well as Michaelis (12), have found that cobalt forms a complex salt with histidine which combines irreversibly with oxygen. Thus, he also attributes the toxic effect of cobalt to an inhibition of cellular oxidation, perhaps by the formation of an oxygen-binding cobalt-histidine complex in the cell.

The purpose of the present investigation was to determine the possible effects of three of the previously mentioned substances, cysteine, methionine, and histidine, on the production of polycythemia by cobalt.

EXPERIMENTAL

Weanling, male, albino rats of the Connecticut Agricultural Experimental Station strain, weighing 40 to 50 gm., were used. They were housed in individual cages and were fed a synthetic basal ration having the following percentage composition: casein 20.0, sucrose 10.0, white corn dextrin 40.0, Crisco 25.7, Wesson's (13) salt mixture 4.0. Synthetic vitamin supplements were incorporated in the foregoing basal diet in the following amounts (in mg. per 100 gm. of diet): thiamine 1, riboflavin 2, pyridoxine 1, niacinamide 2, calcium pantothenate 4, inositol 200, p-aminobenzoic acid 60, folic acid 2, biotin 0.001, and 2-methyl-1,4-naphthoquinone 0.4. In addition, vitamins A, D, and E were supplied as haliver oil with viosterol fortified with α-tocopherol (100 mg. per 50 cc. of oil). 3 drops were administered individually to each rat twice weekly.

The rats were divided into five groups of ten animals each and were given supplements to the basal diet (per kilo of diet) as follows: (1) control, unsupplemented basal diet; (2) cobalt only (0.477 gm. of recrystallized $CoSO_4 \cdot 7H_2O$); (3) cobalt plus cysteine (1.56 gm. or 4.68 gm. of L-cysteine hydrochloride); (4) cobalt plus histidine (6.25 gm. of L-histidine monohydrochloride); and (5) cobalt plus methionine 4.44 gm. of DL-methionine). The lower level of cysteine, 1.56 gm. per kilo of diet, is that found by Griffith and coworkers (9) to neutralize the toxic

effect of cobalt (equivalent to 0.477 gm. of $CoSO_4 \cdot 7H_2O$ per kilo of diet) in so far as response in body weight was concerned. The higher level of cysteine, 4.68 gm. per kilo of diet or 3 times the lower level, was used to determine whether a further effect might be obtained from the increased amount. The levels of methionine and of histidine are isomolar with the higher level of cysteine. All animals, with the exception of the controls, received cobalt as cobalt sulfate in an amount (0.477 gm. per kilo of diet) providing approximately 1.0 mg. of cobalt per rat per day, an amount found in earlier studies capable of producing a definite polycythemia.

In order to evaluate the possible effect of cysteine and histidine on the absorption of cobalt from the gastrointestinal tract, as will be discussed later, three additional groups of rats, of ten to fifteen animals each, in which cobalt with cysteine or histidine was given parenterally, were studied. One group (control) was injected with approximately 0.5 mg. of cobalt daily (1 cc. of an aqueous solution containing 250 mg. of recrystallized $CoSO_4 \cdot 7H_2O$ per 100 cc.). The second group was given an equivalent amount of cobalt as the cobalt-cysteine complex (1 cc. of a solution containing 250 mg. of $CoSO_4 \cdot 7H_2O$ + 500 mg. of L-cysteine hydrochloride + 240 mg. of $NaHCO_3$ per 100 cc.). The third group was given the same amount of cobalt as the cobalt-histidine complex (1 cc. of a solution containing 250 mg. of $CoSO_4 \cdot 7H_2O$ + 400 mg. of L-histidine monohydrochloride + 175 mg. of $NaHCO_3$ per 100 cc.). The solutions were injected subcutaneously in each case. The dosage of cobalt employed in these groups, 0.5 mg. of Co per day, was purposely reduced to approximately half that given orally to the preceding groups in order to compensate, partially at least, for the poor absorption of cobalt from the gastrointestinal tract (14). The amount of cysteine hydrochloride used in the preparation of the cobalt-cysteine complex is slightly in excess of the ratio 1:3, which Michaelis and Barron (15) have shown to be the combining ratio in the complex. Similarly, the amount of histidine monohydrochloride used in preparing the cobalt-histidine complex was slightly in excess of that required for a ratio of 1 part of cobalt to 2 of histidine (Michaelis (12)). In each case, the amount of $NaHCO_3$ added was slightly in excess of that needed to neutralize the hydrochloride of the amino acid preparation used. This also served to adjust the pH of the solutions to values at which the desired cobalt complexes form and at which irritation of the tissues by the injected solution was minimized.

Body weights and food consumption were determined weekly on the various groups of animals, and hemoglobin levels were determined biweekly. Hemoglobin was determined on blood obtained from a tail vein by an acid-hematin method with the Coleman spectrophotometer, previously calibrated by the oxygen capacity method. The animals were

followed for periods varying from 12 to 20 weeks, as stated in Tables I and II.

RESULTS AND DISCUSSION

The results obtained are summarized in Tables I to IV. Table I gives the average body weight for the five groups of rats given oral supplementa-

TABLE I

Average Body Weights in Gm of Control Rats and of Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

Group (10 rats each)	Initial weight	Wks on experiment										
		1	2	4	6	8	10	12	14	16	18	20
Control	44	110	162	258	325	376	420	463	493	517	526	542
Cobalt	45	77	98	135	176	205	237	269	289	309	326	347
" + cysteine												
Low level	42	70	110	166	218	256	298	333	364	380	374	395
High level	53	67	91	163	237	289	334	370*				
Cobalt + histidine	43	66	98	155	199	235	280	306	326	347	366	382
" + methionine	42	92	134	218	261	293	333	359	375	394	410	425

* Group discontinued

TABLE II

Average Hemoglobin (Gm Per Cent) Values for Control Rats and for Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

Group	Initial	Wks on experiment									
		2	4	6	8	10	12	14	16	18	20
Control	10 5	12 8	13 3	14 7	14 8	14 8	15.2	15 7	15 6	15 6	15 5
Cobalt	11 5	12 5	15 5	16 0	17 3	18 6	19 0	19 6	19 6	19.7	20 4
" + cysteine											
Low level	10 6	14.4	15.7	15 7	16 5	17 3	17 6	17 0	17 7	17 6	17 3
High level	10 9	13 5	14.3	15 0	16 3	16 7	17 1*				
Cobalt + histidine	11 3	14.0	16.0	16 5	16 8	17 9	18 2	17 9	18 2	18 2	18 2
" + methionine	10 5	12 9	16 6	17 2	17 5	19 2	19 8	19 5	19 9	19 9	19 9

* Group discontinued

tion. It is evident that the control rats fed the synthetic basal ration grew at a satisfactory rate. The animals given cobalt, on the other hand, showed an inhibition of growth, reaching an average weight of only 347 gm. in 20 weeks as compared to 542 gm. for the controls. The administration of either cysteine, histidine, or methionine with cobalt improved the growth rate considerably, although it was still less than that of the

control animals. The rats given the higher level of cysteine showed somewhat higher average body weight during the 12 weeks they were observed than did the animals receiving the lower dosage. Such a result was not unexpected.

The average data for the daily food consumption (not given in the tables) for the various groups, indicated that the cobalt-treated rats ate more food per 100 gm. of body weight than did the control animals. The average group values for the 20th week of the study were as follows (gm. of food intake per day per 100 gm. of body weight): controls 2.5, cobalt only 3.4, cobalt + cysteine 3.2, cobalt + histidine 3.6, cobalt + methionine 3.2. These data suggest that cobalt decreases the retention or utilization of some dietary constituent or constituents, since the animals ingested more food per unit (100 gm.) of body weight. An improvement in "food utilization" evidently occurred in the rats given either cysteine, methionine, or

TABLE III

Statistical Analysis of Hemoglobin Data*

Group	Average hemoglobin	Standard deviation	Probable error of mean	Probable error† of difference beween means
Control..........	15.6	±0.17	±0 08	
Cobalt...............	19.7	±1.23	±0.24	
" + cysteine, low level..	17.6	±1 60	±0.32	±0.40
" + histidine............ .	18.2	±1 11	±0 23	±0.33
" + methionine.......	19.9	±1.23	±0 25	±0.35

* The values are those for the 18th week of the experiment.

† Comparison made with group given cobalt alone as the supplement.

histidine as a supplement to cobalt. It is interesting that the above values for food intake correspond, inversely, with the terminal average body weights shown in Table I.

Table II gives the average biweekly hemoglobin values. The controls show the normal progressive increase with age, reaching a constant adult level of about 15.6 gm. per cent. The cobalt-fed rats, on the other hand, developed a typical polycythemia as evidenced by the final hemoglobin value of 20.4 gm. per cent. The addition of cysteine, particularly at the higher level, lessened the increase of the hemoglobin values above the controls, as did histidine to a lesser extent. Methionine, on the other hand, had no noticeable effect. This was rather surprising in view of the favorable effect of methionine on the growth and food utilization of the cobalt-fed animals. A statistical analysis of the data, Table III, shows that the effects of cysteine and, to a lesser extent, of histidine, are highly

significant, whereas that of methionine is not, as is also evident from a gross inspection of the data.

Since cobalt forms insoluble complexes with cysteine and with histidine, it appeared possible that these two substances might prevent the production of polycythemia by cobalt by merely decreasing the absorption of cobalt from the gastrointestinal tract. Therefore, three additional groups of rats were studied. They received subcutaneously 0.5 mg. of cobalt, either as cobalt sulfate or as the cobalt-cysteine or cobalt-histidine complex each day for a period of 12 weeks. As was found in the group of rats given oral supplementation, the injection of cobalt sulfate decreased the rate of growth (data not included) as compared with that of the controls. Much less inhibition of growth was observed in the groups given the cobalt-cysteine or cobalt-histidine complex, particularly the former. The data on hemoglobin are recorded in Table IV. It is evident that injected cobalt

TABLE IV

Average Hemoglobin Values for Controls and Cobalt-Injected Rats

Group	No. of rats	Wks. on experiment						
		0	2	4	6	8*	10	12 (standard deviation)
Control	10	13.3	14.7	14.8	15.2	15.7	15.6	15.6 ± 0.17
CoSO₄	10	13.5	16.5	18.9	20.1	18.6	18.7	19.5 ± 0.97
Cobalt-cysteine complex	10	14.3	14.8	15.3	15.3	15.6	16.0	16.1 ± 1.11
Cobalt-histidine complex	15	13.2	16.5†	18.7†	20.0†	18.2	18.5	19.4 ± 0.82

* Daily injections omitted for 5 days during the 7th week.

† The cobalt-histidine complex solution injected during this interval did not contain added $NaHCO_3$, as described for the preparation of this solution in the text.

sulfate produced a polycythemia, whereas the cobalt-cysteine complex in an equivalent amount of cobalt did not increase the hemoglobin level significantly above that of the controls. The average hemoglobin values of the group receiving the cobalt-histidine complex, on the other hand, differed little from that to which cobalt sulfate was given and a typical polycythemia resulted. This observation is in general agreement with that in the group given cobalt with histidine orally, although some inhibition of the production of polycythemia by cobalt was observed in the latter case. This may be due to the fact that the ratio of histidine to cobalt was much greater in the orally supplemented group than was possible in the injected group. These data therefore indicate that the inhibition of the polycythemia by cysteine because of a possible impairment of cobalt absorption from the gastrointestinal tract cannot be a determining factor, since cobalt bound as a cysteine complex and administered parenterally in an amount comparable to that given orally likewise does *not* produce a polycythemia.

At least two explanations of the effect of cysteine and, to a lesser extent, histidine in preventing the production of polycythemia by cobalt appear possible. One is that these two substances, by forming complex compounds, may increase the excretion of cobalt and thus lessen its ability to produce polycythemia. However, such an explanation seems rather unlikely, because other substances such as choline and, under certain conditions, methionine also may form complexes with cobalt analogous to those of cysteine and histidine and thus presumably likewise increase cobalt excretion in the urine. Furthermore, under ordinary circumstances the excretion of parenterally administered cobalt is rapid and almost complete within 36 hours (14). Moreover, Stare and Elvehjem (16) have shown by analysis that at the height of the polycythemia there are present only 40 to 50 γ of cobalt in the entire body of the rat.

Another explanation, the more likely in our opinion, is that the administered cysteine, and possibly also histidine, combines with cobalt to form insoluble or inert complexes in the organism, thus preventing its subsequent "blocking" of sulfhydryl and perhaps other groups active in cellular respiration, which, in turn, would prevent the development of a compensatory polycythemia. Such an interpretation is in accord with the observation (Burk, personal communication) that "the relative affinities of cobalt for the naturally occurring amino acids are, in decreasing order, cysteine, histidine, and then the others." Cobalt also has a relatively low affinity for choline. This would thus satisfactorily explain the failure of methionine and choline to prevent the production of polycythemia by cobalt. However, further work will be required to answer these questions in a positive manner.

SUMMARY

The oral administration of 1.0 mg. of cobalt as cobalt sulfate to rats fed a synthetic basal diet produces a sustained polycythemia, an inhibition of growth, and impairment in food utilization.

Supplementation of the cobalt-containing diet with cysteine inhibits the production of the polycythemia. Histidine has a similar effect but to a lesser extent. Methionine has no detectable effect when fed in equivalent amounts.

Parenterally administered cobalt sulfate (0.5 mg. of cobalt per day) likewise produces a marked polycythemia, whereas an equivalent amount of cobalt as the cobalt-cysteine complex does not. Histidine injected with cobalt as the cobalt-histidine complex has less effect in preventing the development of polycythemia.

It is proposed that cysteine inhibits the production of polycythemia by cobalt by the formation in vivo of an inert cobalt-cysteine complex. Histi-

dine may act in a similar manner but the cobalt-histidine complex is more active (less completely formed) than the cobalt-cysteine complex at the pH values existing in the animal organism.

The suggestion is made that cobalt may produce polycythemia by binding sulfhydryl or perhaps other groups active in cellular respiration, thus leading to a simulated cellular anoxia and, in turn, to a compensatory polycythemia.

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THE USE OF ION EXCHANGE RESINS IN THE ISOLATION OF BLOOD GROUP A-SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN*

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Ion exchange resins (1) have been used in the investigation of A substance hydrolysates (2), but apparently no attempt has been made to determine whether they can be profitably used in the isolation or purification of undegraded A substance[1] from sources such as hog gastric mucin. Studies along these lines are reported in this communication.

EXPERIMENTAL

Ion Exchange Resins—De-Acidite (The Permutit Company, New York), washed on a 40 mesh screen with distilled water, was treated several times with aqueous 4 per cent sodium carbonate and repeatedly washed with distilled water, by suspension and decantation, until the supernatant liquid was colorless and had a pH of 8.0 or less. The resin collected on a suction filter contained 70 to 80 per cent water. 1 gm. (dry weight) of the resin contributed less than 0.05 milliequivalent of base to 75 ml. of distilled water when the suspension was allowed to stand, with frequent shaking, for 2 days at 25°. Under the same conditions 1 gm. (dry weight) of the resin removed 95 per cent of the hydrochloric acid from 75 ml. of a 0.066 M solution and 85 per cent from 75 ml. of a 0.093 M solution.

Amberlite IR-4 (The Resinous Products and Chemical Company, Philadelphia) prepared as described above, contained approximately 35 per cent water, and 1 gm. (dry weight) of the resin removed more than 95 per cent of the hydrochloric acid present in 25 ml. of a 0.166 M solution.

Amberlite IR-100 (The Resinous Products and Chemical Company), treated with 1 per cent hydrochloric acid and washed free of chloride, gave a product containing approximately 35 per cent water.

A Substance Preparations—A 2 per cent suspension of hog gastric mucin granules (Wilson), adjusted to pH 4.4 to 4.5 with glacial acetic acid, was

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[1] Defined as a substance effective in inhibiting isoagglutination of human blood group A cells by group B serum and also effective in inhibiting lysis of sheep erythrocytes by human blood group A cell immune rabbit sera.

centrifuged twice in the open bowl of a Sharples centrifuge at 20,000 R.P.M. The centrifugate was used either directly or after ethanol fractionation (3, 4).

Procedure

Sufficient exchange resin was added, unless otherwise indicated, to a 1 to 2 per cent aqueous solution of the A substance preparation to provide 4 to 5 gm. (wet weight) of freshly washed resin for each gm. of dissolved solid. The suspension was stirred for 2 to 3 hours at 5°, filtered, and the operation
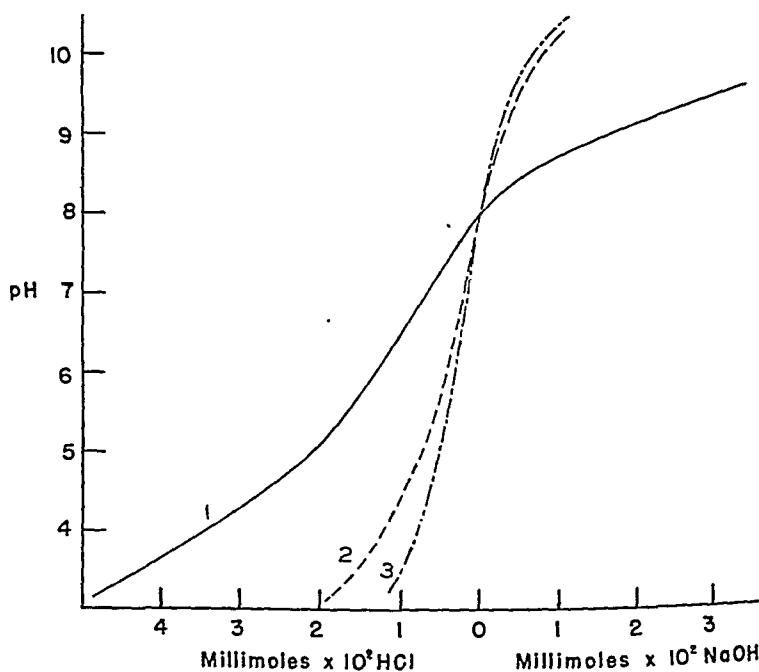

FIG. 1. Titration curves of A substance preparations. Curve 1, Fraction 130 Curve 2, Fraction 128; Curve 3, Fraction 141.

repeated. The resulting solution was either lyophilized or subjected to further treatment with a different exchange resin. Equivalent N-acetyl glucosamine contents and inhibition of hemolysis titers were determined as described previously (4, 5). Titration curves were determined by adding sufficient hydrochloric acid to 50 mg. of solid in 5 ml. of water to decrease the pH to 3.0 or less and then titrating with 0.040 M sodium hydroxide. Since an inflection point was observed at pH 8, the data are plotted in Fig. 1 with reference to the number of millimoles of acid or base required to change the pH of the above solution from pH 8 to some other pH.

DISCUSSION

Within limits the equivalent N-acetylglucosamine content (5) of an A substance preparation can be taken as an index of the A substance activity of the preparation, evaluated in terms of inhibition of isoagglutination or inhibition of hemolysis (4, 5). Preliminary experiments based upon the determination of the equivalent N-acetylglucosamine content of an A substance solution before and after treatment with a solid adsorbent previously equilibrated with water indicated that of a number of adsorbents tested the anion exchange resins De-Acidite and IR-4 and the cation exchange resin IR-100 were sufficiently promising to warrant further investigation.[2]

The results obtained by treating an aqueous solution of hog gastric mucin with the above ion exchange resins, either singly or in combination, are given in Table I. It is noteworthy that successive application of De-Acidite and IR-100 gave a preparation which was as active in the hemolysis test[3] as those obtained from mucin by ethanol fractionation (3, 4). The separation of a resin-treated mucin solution into a clear supernatant and a less active viscous precipitate is reminiscent of the behavior observed when A substance preparations obtained from mucin by ethanol fractionation are electrodialyzed (4).[4] Although resin treatment will give products[3] as active as those obtained by ethanol fractionation, there is no evidence that further treatment of the product with any of the above resins will lead to more active preparations such as those obtained by a combination of ethanol fractionation and electrodialysis.[4]

A substance preparations of varying purity, obtained from hog gastric mucin by ethanol fractionation or electrodialysis, were subjected to resin treatment and, in contrast to the experience with mucin, only in one instance was an increase in activity observed (Table II). In the isolation of A substance from hog gastric mucin, it has been noted that an increase in A substance activity is usually accompanied by an increase in the equivalent N-acetylglucosamine content of the preparation (4).[4] However, with A substance preparations containing more than 10 per cent equivalent N-acetylglucosamine, the equivalent N-acetylglucosamine content may not necessarily increase with A substance activity upon further purification and in some instances may actually decrease.[4] This behavior is not unexpected, since it has been pointed out that blood group-specific substances other than the A substance contain alkali-labile bonds involving N-acetylglucosamine

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[2] It is possible that with a different test or under different conditions other adsorbents may have been selected.

[3] Comparable titers were also obtained with the inhibition of the isoagglutination test which would indicate that resin treatment does not cause the degradation of A substance.

[4] Holzman, G., and Niemann, C., unpublished experiments.

TABLE I

Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Hog Gastric Mucin Suspension after Successive Treatments with Several Exchange Resins

Description of fraction*	Fraction No. of product	Yield	Inhibition of hemolysis titer†	Equivalent N-acetyl-glucos-amine content‡
Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate	135	72% from mucin	0.19 ± 0.02	7.6
Fraction 135 treated twice with De-Acidite	136	83% from No. 135	0.16 ± 0.01	8.9
Fraction 136 treated twice with IR-100; clear supernatant and viscous ppt. obtained upon standing 24 hrs.; separated	137 (ppt.)	50% from No. 136	0.11 ± 0.01	10.8
	138 (supernatant)	23% from No. 136	0.105 ± 0.005	11.6
Fraction 137 treated twice with IR-4	139	100% from No. 137		11.2
Fraction 138 treated twice with IR-4	140	100% from No. 138		11.7
Fraction 136 treated twice with IR-100 and twice with IR-4	141	67% from No. 136	0.12 ± 0.01	11.2
Fraction 135 treated alternately 4 times with De-Acidite and IR-100; allowed to stand 4 days; turbid supernatant and viscous ppt. obtained; separated	152 (supernatant)	32% from mucin	0.14 ± 0.02	10.5
	154 (ppt.)	20% from mucin	0.19 ± 0.02	9.2
Fraction 152 treated with De-Acidite and IR-100	153	88% from No. 152	0.12 ± 0.01	11.2

* 4 to 5 gm. (wet weight) of the exchange resin per gm. of material treated were used for each treatment, except in the preparation of Fractions 167 and 168, in which 8 gm. of De-Acidite per gm. were used for each treatment, and in the preparation of Fractions 152, 154, and 153, in which 0.5 gm. portions of De-Acidite and IR-100 per gm. were used for each treatment.

† Micrograms of test substance present in system in which sheep erythrocytes are 50 per cent hemolyzed by an anti-human A cell immune rabbit serum. The titers reported are the average of two or three determinations and are reported together with the average deviation of the several determinations.

‡ Expressed as equivalent per cent of N-acetylglucosamine in test substance (5). The analytical data reported are the mean results of duplicate or triplicate analyses. The absolute deviation was in no case larger than 0.3 per cent (equivalent N-acetyl glucosamine).

(5, 6), and it is known that A- and O-specific substances can be separated at least in part, by relatively simple fractionation procedures (7).[4]

TABLE II

Inhibition of Hemolysis Titer and Equivalent N-Acetylglucosamine Content of Some Blood Group A-Specific Substance Preparations after Treatment with Exchange Resins

Description of fraction*	Fraction No. of product	Yield	Inhibition of hemolysis titer†	Equivalent N-acetyl-glucosamine content‡
Aqueous suspension of hog gastric mucin granules centrifuged twice at pH 4.4; centrifugate (Fraction 135) fractionated with ethanol; material soluble in 40% (by volume) ethanol, insoluble in 65% (by volume) ethanol	143	22% from mucin	0.11 ± 0.01	10.6
Fraction 143 treated twice with De-Acidite	144	77% from No. 143	0.105 ± 0.015	12.0
Fraction 144 treated twice with IR-100	145	100% from No. 144	0.105 ± 0.010	12.3
Fraction 145 treated twice with IR-4	147	100% from No. 145	0.11 ± 0.01	12.1
Aqueous suspension of hog gastric mucin granules; centrifugate fractionated with ethanol; material soluble in 30% (by volume) ethanol, insoluble in 65% (by volume) ethanol; upon reprecipitation, insoluble in 45% (by volume) ethanol; dialyzed, then electrodialyzed	110		0.20 ± 0.02	11.8
Fraction 110 treated twice with De-Acidite	167	74% from No. 110	0.21 ± 0.02	13.4
Fraction 110 treated twice with IR-4	165	91% from No. 110	0.20 ± 0.02	11.8
Same as Fraction 110, except upon reprecipitation, material soluble in 45% (by volume) ethanol, insoluble in 65% (by volume) ethanol; dialyzed, then electrodialyzed	126		0.088 ± 0.005	10.4
Fraction 126 treated twice with De-Acidite	168	60% from No. 126	0.070 ± 0.005	11.9
Fraction 126 treated twice with IR-4	166	88% from No. 126	0.086 ± 0.005	10.5

See the corresponding foot-notes to Table I.

The effectiveness of De-Acidite and IR-100 in reducing the " buffering capacity" of mucin solutions and partially purified A substance preparations is clearly illustrated in Fig. 1. The anion exchange resin IR-4 was found to be markedly inferior to De-Acidite in this respect. Analysis of Fractions

135, 136, and 137 for total nitrogen, amino nitrogen, and amino acid nitrogen (Table III) revealed that De-Acidite was instrumental in removing acidic nitrogenous non-blood group-specific substances containing little or no amino nitrogen, whereas the substances removed by IR-100 contained substantial amounts of amino and amino acid nitrogen.   At least part of the materials removed by De-Acidite are non-dialyzable.[5]   It is interesting to note that in a centrifuged mucin solution approximately 20 per cent of the solids are not precipitated by 66 per cent ethanol, whereas approximately 30 per cent are removable by successive treatment with De-Acidite and IR-100.

It has been observed that many A substance preparations are contaminated by non-blood group-specific substances[6] exhibiting marked specific absorption in the 260 to 270 mμ region (8).   An A substance preparation (Fraction 110) containing a substantial amount of the "260 mμ component" ($E_{1\ cm.}^{1\%}$ at 260 mμ = 13.2) was treated with De-Acidite and the resulting preparation (Fraction 167) was found to have a value of $E_{1\ cm.}^{1\%}$ at 260 mμ

TABLE III

Nitrogen Content of A Substance Preparations

Fraction No.	Total N	Amino N	Amino acid N
	per cent	*per cent*	*per cent*
135	8.4	1.9	1.0
136	7.3	2.0	1.2
137	6.4	0.16	0.09

of 3.6.   Treatment with IR-100 or IR-4 caused little or no decrease in extinction.   The fact that De-Acidite was effective and IR-4 was relatively ineffective in removing the "260 mμ component" would indicate that the removal of this component by De-Acidite is not a simple anion exchange. An explanation of the mode of action of De-Acidite leading to the loss of the "260 mμ component," a gain in the equivalent N-acetylglucosamine content, and no significant change in A activity must await the accumulation of additional data.

SUMMARY

The treatment of hog gastric mucin with the two ion exchange resins De-Acidite and IR-100 has given apparently undegraded A substance preparations which are as active as those obtained from the same source by

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[5] Bennett, E. L., unpublished data.

[6] While the "260 mμ component" has not been obtained free of A substance, all evidence (8) points to its non-blood group specific character.

ethanol fractionation. This enrichment in A substance is due to the partial removal by these two resins of some of the non-blood group-specific components normally present in hog gastric mucin.

The authors wish to express their indebtedness to Dr. D. H. Brown and Dr. G. Holzman for their assistance in this investigation.

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It has been observed that many A substance preparations are contaminated by non-blood group-specific substances[6] exhibiting marked specific absorption in the 260 to 270 mμ region (8).   An A substance preparation (Fraction 110) containing a substantial amount of the "260 mμ component" ($E_{1\,cm.}^{1\%}$ at 260 mμ = 13.2) was treated with De-Acidite and the resulting preparation (Fraction 167) was found to have a value of $E_{1\,cm.}^{1\%}$ at 260 mμ

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Fraction No.	Total N	Amino N	Amino acid N
	per cent	*per cent*	*per cent*
135	8.4	1.9	1.0
136	7.3	2.0	1.2
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SUMMARY

The treatment of hog gastric mucin with the two ion exchange resins De-Acidite and IR-100 has given apparently undegraded A substance preparations which are as active as those obtained from the same source by

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[5] Bennett, E. L., unpublished data.

[6] While the "260 mμ component" has not been obtained free of A substance, all evidence (8) points to its non-blood group specific character.

STUDIES ON THE NATURALLY OCCURRING PENICILLINS

II. PRECIPITATION OF CRYSTALLINE AMMONIUM PENICILLINS

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It has been disclosed (1) that there are several antibiotics of the penicillin class and that all are salts of acids having the empirical formula $C_9H_{11}O_4SN_2$—R. Among the naturally occurring penicillins are those in which R is respectively benzyl, n-heptyl, 2-pentenyl, and p-hydroxybenzyl. Commercial penicillin may be any one or a mixture of these penicillins, depending on the method of production and isolation. The alkali and alkaline earth salts of penicillin are extremely soluble in water. The only known methods of crystallizing penicillin salts such as sodium, potassium, and ammonium are by the use of organic solvents, usually in the presence of minute amounts of water. The penicillins, as usually isolated, are associated with colored and odoriferous materials which are sometimes very difficult to remove by simple methods.

We have found that by adding soluble ammonium salts to moderately concentrated solutions of various penicillin salts it is possible to crystallize a high proportion of the penicillin as ammonium penicillin. It is possible to prepare material with little color or odor by the procedure. The penicillin used should be in a concentration equivalent to at least 200,000 units per ml. as tested by the turbidimetric method with *Staphylococcus aureus* strain H (2). It should also have a quality of 1000 units per mg. or better when dried. With more crude or dilute starting material, the results are dependent on the nature and quality of the penicillin. In our hands, it has mattered little whether the original penicillin was of the benzyl, pentenyl, or heptyl species, as long as sufficient ammonium salt was added. With any given salt concentration, the solubilities of the various penicillin species differ considerably. This is illustrated in Table I, which gives the solubilities of ammonium benzyl- and heptylpenicillins in saturated ammonium chloride solution.

The ammonium salts which seem to be most useful are the sulfate, chloride, bromide, and acetate. However, a number of others have been used and a comparison of the yields of ammonium penicillin after addition of a solution of pure sodium benzylpenicillin having 300,000 units per ml. to various strong ammonium salt solutions is given in Table II.

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The ammonium penicillin precipitate is washed with fresh strong ammonium salt solution and is of course contaminated with this ammonium salt. When a pure ammonium penicillin is desired, we have found ammonium sulfate to be the salt of choice for the precipitation because of its easy removal. By treating the air-dried precipitate with the proper amount of dioxane containing 10 per cent water, the ammonium sulfate remains undissolved. After filtration, the solution is then treated with 4 volumes of dry dioxane to precipitate the pure ammonium penicillin. When ammonium acetate is used as the precipitant, the product may be dried under a high vacuum to remove the ammonium acetate and give a crystalline ammonium penicillin of high potency. As both ammonium chloride and bromide present some difficulty in their separation from ammonium penicillin, their use is limited to instances in which other salts are

TABLE I

Solubilities of Ammonium Benzylpenicillin and Heptylpenicillin in Saturated Ammonium Chloride

Penicillin	Amount at start	In mother liquor	Insoluble	Heptyl-	Benzyl-	Solubility in saturated NH$_4$Cl
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	*mg. per ml.*
Heptyl-	120	8	112	100	0	0.8
" Heptyl- Benzyl-	60 60	50	70	95	5	5
Heptyl- Benzyl-	60 120	80	40	Not determined		8
Heptyl- Benzyl-	120 60	50	110	85	15	5
"	140	118	23	0	100	11.8

desired. In these cases, the crude ammonium salt is dissolved in water, acidified at 0–4°, and shaken with ether. The ether solution is then treated with an alkali or alkaline earth bicarbonate solution. On evaporating this aqueous solution in a high vacuum, a high quality penicillin salt is obtained which in many cases may be crystallized.

The method is being extended to the use of salts other than ammonium and shows considerable promise.

EXPERIMENTAL

In determining the solubilities of ammonium benzyl- and heptylpenicillins in saturated NH$_4$Cl (Table I) the amount of penicillin used was warmed to 40–45° with 10 ml. of saturated ammonium chloride (pH 6.8) and then allowed to stand at room temperature for 1 hour. After filtration, the

insoluble material was dissolved in water and the amount of penicillin was determined by optical rotation. The same method was used on the soluble portion. For this determination $[\alpha]_D^{25} = +300° \pm 5°(c = 1$ in water) was used as the value for pure sodium benzylpenicillin and $[\alpha]_D^{25} = +285° \pm 5°$ for the pure sodium heptylpenicillin. The proportion of insoluble ammonium benzylpenicillin was determined by means of the Beckman spectrophotometer (3).

For the determination of the relative precipitating action of the various ammonium salts (Table II) 1 gm. of pure sodium benzylpenicillin crystals was dissolved in 1 ml. of water, treated with 5 ml. of the concentrated salt solution with stirring, and the precipitate washed with 5 ml. of the same concentrated solution. The concentration of penicillin in the precipitate

TABLE II

Precipitation of Ammonium Benzylpenicillin by Salts

Ammonium salt	Amount in ppt.	In mother liquor	Concentration of ammonium salt per 100 ml. solution
	mg.	*mg.*	*gm.*
Acetate	965	35	60
Bromide	709	291	55
Chloride	887	113	30
Formate	980	20	70.4
Iodide	0	1000*	83.3
Nitrate	805	195	68.6
Phosphate	980	20	46
Sulfate	998	2	53

All solutions adjusted to pH 6.5 to 7.5 with NH_3 or the corresponding acid.

* Decomposition was very rapid and a slight precipitate dissolved in a short time.

and in the mother liquor was determined by rotation and bioassay as previously discussed.

When 262 ml. of saturated $(NH_4)_2SO_4$ solution, adjusted to pH 6.5 with NH_4OH, were treated, with stirring, with 60 gm. of commercial sodium penicillin assaying 1100 units per mg., the penicillin ammonium salts soon began to crystallize and within a few minutes crystallization was complete. The magma was filtered and washed with 50 per cent $(NH_4)_2SO_4$ solution until the wash liquor became colorless. After drying in a vacuum oven, 44.1 gm. of product containing 78 per cent ammonium penicillins were obtained. This is 86 per cent of the penicillins contained in the starting material.

In another experiment 400 ml. of crude sodium penicillin liquor assaying

300,000 units per ml. were treated with 300 gm. of powdered $(NH_4)_2SO_4$ with stirring. The precipitate was filtered and washed as indicated previously. The dry cake was stirred with 400 ml. of dioxane containing 10 per cent of water. After filtration, the solution was treated with 1200 ml. of dry dioxane. The ammonium penicillin was precipitated and after filtering, washing, and drying 48.7 gm. of mixed ammonium penicillin were obtained. This was 67 per cent of the penicillin in the starting material. The product was shown to be essentially all ammonium penicillin by its rotation ($[\alpha]_D^{25} = +294°(c = 1$ per cent in water)) and its biological assay of 1650 units per mg.

When a crude sodium penicillin solution containing essentially all of the penicillin as the benzyl species was treated in the same manner, there were obtained 12 gm. of ammonium salt from 100 ml. of liquor assaying 300,000 units per ml. This product was pure ammonium benzylpenicillin as shown by analysis, rotation, and bioassay. The optical rotation was $[\alpha]_D^{25} = +298°$ ($c = 1$ per cent in water) and the bioassay 1650 units per mg.

$C_{16}H_{21}O_4SN_2$. Calculated. C 54.70, H 5.98, N 11.97, S 9.12
Found.   " 54.62, " 6.15, " 11.90, "9.31

Ammonium acetate (172.5 gm.) was adjusted to pH 6.5 with NH_4OH and made up to 330 ml. with water. This solution was treated with 100 gm. of dry commercial penicillin assaying 1000 units per mg. Stirring was continued for about 15 minutes to crystallize the penicillin. The ammonium penicillin was then filtered and washed with 50 per cent ammonium acetate solution. On drying in a high vacuum, the excess ammonium acetate volatilized and 30.8 gm. of ammonium penicillin, having $[\alpha]_D^{25} = +296$ ($c = 1$ per cent in water) and a bioassay of 1620 units per mg., were obtained. This is 50 per cent of the penicillin in the starting material.

To 770 ml. of a crude sodium benzylpenicillin concentrate containing 300,000 units per ml. were added 230 gm. of NH_4Cl with stirring. The mixture was filtered and washed with saturated ammonium chloride. The vacuum-dried cake contained 75 per cent of the original penicillin as crystalline ammonium salt contaminated with ammonium chloride. The crude ammonium salt was dissolved in 2 liters of water, adjusted to pH 2 with H_3PO_4, and extracted three times with 2 liter portions of ether. The ether extract was then stirred with 2 per cent $NaHCO_3$ solution. Additions of fresh $NaHCO_3$ solution were made until pH 7 was reached. This solution, on evaporation in a high vacuum, yielded amorphous sodium benzylpenicillin, which was dissolved in 400 ml. of 10 per cent aqueous dioxane and precipitated by the addition of 1200 ml. of dry dioxane. The resulting crystalline salt had $[\alpha]_D^{25} = +299°$ and a bioassay of 1660 units per mg.

$C_{16}H_{17}O_4N_2SNa$. Calculated. C 53.93, H 4.81, N 7.86, S 9.00, Na 6.46
Found   " 53.97 " 5.01, " 7.86, "9.03, " 6.43

We wish to thank Dr. J. A. Means for performing the microanalyses. We also wish to acknowledge the interest and encouragement of Dr. Richard Pasternack and Mr. Howard Hedger.

SUMMARY

A new method for the purification of penicillin, which gives an essentially colorless, odorless ammonium penicillin, has been devised. Other pure salts may be made from the ammonium salts by known methods. The method appears to be independent of the species of penicillin present.

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FRACTIONATION OF SERUM INTO ALBUMIN AND α-, β-, AND γ-GLOBULIN BY SODIUM SULFATE

By ANDRE C. KIBRICK AND MURIEL BLONSTEIN

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The values of the albumin and total globulin of serum determined with 22.4 per cent sodium sulfate by the method of Howe (1) are better correlated with the values of one fraction containing the albumin and α-globulin and of another containing the β- and γ-globulins (2). However, 19 per cent sodium sulfate seems to separate these fractions more efficiently, since it yields results which agree very well with the corresponding fractions determined by electrophoresis (3). Apparently the precipitation of all of the globulin fractions of human serum requires 26 to 27 per cent sodium sulfate (3, 4). The data of Majoor (4) indicate that most of the γ-globulin is precipitated by 15 per cent of the salt. This is consistent with the findings of Gutman *et al.* (5) that the γ-globulin fraction is only partially precipitated at a concentration of 13.5 per cent of the salt, while 17.4 per cent removes a significant amount of β-globulin in addition to the γ fraction. Moreover, 15 per cent sodium sulfate is approximately equivalent to 0.33 saturated ammonium sulfate in respect to salting out. Jager and Nickerson found a good correlation between the amounts of protein precipitated by the latter and the values of γ-globulin estimated by electrophoresis (6). Hence, it occurred to us that human serum can be analyzed for albumin and for all of the globulin fractions by determining the protein precipitated with 15, 19, and 26 per cent sodium sulfate. The present study was undertaken to determine whether the values estimated by this simple method are consistent with the results of other methods.

EXPERIMENTAL

A series of thirteen sera, obtained from patients with miscellaneous clinical conditions in this hospital, was analyzed by fractionation with sodium sulfate. In addition, the albumin and total globulin were determined with methyl alcohol by the method of Pillemer and Hutchinson (7), and the γ-globulin fraction was determined with serum of rabbits immunized to this protein. Another series of sera and plasmas of known protein composition determined by electrophoretic analysis was obtained from other laboratories.[1] These were analyzed by fractionation with salt.

The fractionation was made by adding 0.5 ml. of serum or plasma to 10 ml. of 15.75, 19.90, and 27.20 per cent sodium sulfate at 37°. About 10 mg. of Hyflo Super-Cel were added from a scoop made from glass tubing, and the mixtures were allowed to stand in the incubator at 37° for 1 hour.[2] The precipitates were then filtered in covered funnels in the incubator with Whatman No. 50 filter paper, 9 cm. in diameter. Portions of the clear filtrates were added to the biuret reagent, and their protein content was determined by the method of Kingsley (8) as modified by Kibrick and Clements (9).

The biuret reaction was also utilized to determine the total protein of the sera and the albumin in the methyl alcohol filtrates from the method of Pillemer and Hutchinson, as described previously (9).

Serum immune to human γ-globulin was obtained by injecting rabbits twice weekly by ear vein with 1 to 2 ml. of 1 per cent of the protein in glycine solution[3] containing 0.05 per cent aluminum ammonium sulfate. After about 6 weeks and a period of 6 days without injections, the animals were bled from the heart and the serum was prepared with 0.01 per cent sodium merthiolate. Traces of antibodies to the other serum proteins were removed by absorption with a solution of human albumin[4] and with a mixture of the α- and β-globulins.[5] The determinations were made by adding 2 ml. of the immune serum in centrifuge tubes to 1 ml. of human serum which had been diluted 50 to 150 times with 0.9 per cent sodium chloride. The tubes were allowed to stand in a water bath at 40° for 2 hours and then placed in the refrigerator until the next day. The immune precipitates were centrifuged and washed twice with 3 ml. of cold 0.9 per cent sodium chloride. They were then dissolved in dilute alkali and their content of

[2] We have found that the amount of soluble protein was essentially the same when the precipitates were filtered from 30 minutes to as long as 24 hours after mixing. The filtrates were frequently turbid, however. The use of Super-Cel produces clear filtrates in almost every case and does not affect the results.

[3] Prepared from immune serum globulin, Cutter Laboratories, containing 17 per cent of the protein in 0.3 M glycine.

[4] We wish to thank Dr. F. F. Johnson of the Cutter Laboratories, Berkeley, for samples of human albumin and γ-globulin which do not contain other electrophoretic components.

[5] We are indebted to Professor E. J. Cohn for a concentrated fraction of the α- and β-globulins from human serum.

nitrogen was determined by the micro-Kjeldahl method. Several standard tubes containing a dilute solution of γ-globulin[4] were run with each series of determinations. The concentration of γ-globulin was calculated from a standard curve prepared from the results of serial dilutions of a weighed amount of the protein.

RESULTS AND DISCUSSION

Table I shows that the values of albumin and total globulin determined with sodium sulfate agree quite well with those determined with methyl

TABLE I

Comparison between Values Derived from Fractionation with Sodium Sulfate and Those from Precipitation with Methyl Alcohol and with Immune Serum

The results are expressed in gm. per 100 ml.

Sample No.	Total protein	Albumin		Globulin fractions			
		Salt	Alcohol	With sodium sulfate			Precipitin
				α-	β-	γ-	γ-
1	7.16	3.74	3.57	1.04	0.61	1.77	1.6
2	6.98	3.87	4.02	0.75	0.82	1.54	1.7
3	6.98	3.23	3.42	1.30	0.66	1.79	1.8
4	6.70	3.71	3.63	0.70	1.05	1.24	1.2
5	4.78	1.79	1.21	0.93	0.56	1.5	1.2
6	8.00	3.99	4.12	0.86	0.68	2.47	2.5
7	7.49	3.93	4.25	0.99	0.61	1.96	2.2
8	8.14	3.07	2.95	1.05	0.76	3.26	3.0
9	7.04	3.44	3.71	1.18	0.57	1.85	1.5
10	7.04	3.24	3.48	1.51	0.77	1.52	1.3
11	7.15	3.43	3.21	0.83	0.47	2.42	2.3
12	7.95	3.70	3.86	1.25	0.70	2.30	2.2
13	4.02	1.02	1.38	1.35	0.57	1.08	1.3

alcohol. This is further confirmation that 26 per cent sodium sulfate effects a reliable separation in the serum of subjects with a variety of clinical conditions. The values of the γ-globulin fraction also agree quite well with the results obtained by precipitation with immune serum. Kabat *et al.* have determined the γ-globulin in cerebrospinal fluid by the latter method (10), but it does not seem so convenient for routine use. Table II shows that the protein precipitated with 15 per cent sodium sulfate is in satisfactory agreement with the γ-globulin fraction estimated by electrophoretic analysis.

We have found that the amount of protein precipitated with 19 per cent sodium sulfate in about 100 different sera is only slightly less than that precipitated with 22 per cent salt by the method of Howe. In many

instances the results were almost identical, but there was a suggestion that better agreement is possible between duplicate determinations with the smaller concentration. There seems to be a definite break in the precipitation of protein at about 19 per cent, which the data of Milne (3) indicate is equal to the sum of the β- and γ-globulin fractions. If this is correct, the amounts of the α and β fractions, derived from this value and from the values of total globulin and of the γ fraction, are also correct. Table II shows that they are in agreement with those found by electrophoresis.

TABLE II

Comparison between Results of Fractionation with Sodium Sulfate and Those from Electrophoresis

The results are expressed in gm. per 100 ml.

Sample No.	Salt fractionation				Electrophoresis			
	Albumin	Globulin			Albumin	Globulin		
		α-	β-	γ-		α-	β-	γ-
1				0.8	3.2	0.8	1.1	0.9
2				5.3	1.7	0.8	0.5	4.5
3				0.6	3.8	1.0	0.8	0.7
4				1.0	3.4	1.4	1.2	1.2
5				1.2	4.5	1.2	1.0	1.2
6	2.8	1.0	1.1	1.6	3.0	0.9	1.0	1.5
7	2.8	1.0	1.2	1.2	3.0	0.8	1.0	1.3
8	2.8	1.0	0.9	1.5	2.8	0.8	1.0	1.6
9	2.8	0.8	1.2	1.6	2.7	0.7	1.2	1.7
10	2.3	1.3	0.7	1.1	2.5	1.1	0.7	0.9
11	3.0	1.3	0.7	0.8	3.3	1.0	0.7	0.8
12	2.4	1.3	1.0	0.9	2.4	1.5	1.0	0.7
13	2.3	1.1	1.0	1.0	2.2	1.2	0.9	0.7
14	2.1	0.9	0.7	0.7	2.2	0.8	0.6	0.7

We are grateful to Dr. Joseph Felsen, Director of Laboratories and Research, for advice and encouragement.

SUMMARY

1. A simple chemical method is proposed to estimate the fractions of protein in human serum by precipitation with 15, 19, and 26 per cent sodium sulfate. The results of a series of thirteen sera are compared with the values of albumin and total globulin determined with methyl alcohol and with the values of γ-globulin determined by precipitation with immune rabbit serum. The results in another series of fourteen samples are compared with values derived from electrophoretic analysis.

2. Most of the values of albumin, total globulin, and γ-globulin are within 0.2 gm. of those determined with methyl alcohol and with immune serum.

3. The values of albumin and of α-, β-, and γ-globulin also compare favorably with the results of electrophoresis.

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LETTERS TO THE EDITORS

PUTRESCINE AS A GROWTH FACTOR FOR HEMOPHILUS PARAINFLUENZAE*

Sirs:

A requirement for unidentified growth factors by some members of the genus *Hemophilus* has been reported.[1] During a study of the nutritional requirements of one member of this genus, *Hemophilus parainfluenzae* 7901, an amino acid medium was developed which was satisfactory for the assay of an essential factor supplied by a variety of crude materials including yeast and orange juice. Orange juice was chosen for fractionation and a crystalline compound, identified as putrescine, was isolated which completely replaced orange juice when added to the amino acid medium. The isolation was accomplished by the following procedures: (1) adsorption from clarified canned orange juice on Amberlite IR-100-H; (2) elution from the cation exchange resin with 20 per cent HCl after removal of inactive solids with 4 per cent H_2SO_4; (3) concentration of the eluate to small volume, addition of 50 per cent NaOH, and steam distillation; (4) neutralization of steam distillate with 10 per cent H_3PO_4 and precipitation of the dipicrate by addition of sodium picrate solution to the concentrated steam distillate; and (5) repeated recrystallization from hot water. The dipicrate was converted to the dihydrochloride and the dibenzoate by standard procedures. The dibenzoate melted at 178–180°. A mixture with an authentic sample of putrescine dibenzoate (m.p. 178–180°) melted at 178–180°. The melting point of the dipicrate also checked that reported in the literature and gave no depression with an authentic sample of this compound.

Analysis of Dihydrochloride—$C_4H_{12}N_2 \cdot 2HCl$. Calculated, N 17.39; found, 17.43, 17.48.

The essential nature of putrescine for growth of *H. parainfluenzae* is shown in the table. Spermine and spermidine, whose molecules contain the 1,4-diaminobutane residue, are also active. However, a variety of

[1] Bass, A., Berkman, S., Saunders, F., and Koser, S. A., *J. Infect. Dis.*, 68, 175 (1941).

compounds having a structural relationship to putrescine and including ornithine, cadaverine, 1,3-diaminopropane, 1,6-diaminohexane, n-butylamine, and pyrrolidine was found to be totally inactive, as were the polyamines, triethylenetetramine and tetraethylenepentamine. A high

Effect of Putrescine, Spermine, and Spermidine on Growth of Hemophilus parainfluenzae[*]

Orange juice solids		Putrescine		Spermine		Spermidine	
	Turbidity[†]		Turbidity[†]		Turbidity[†]		Turbidity[†]
mg. per 10 cc.		*γ per 10 cc.*		*γ per 10 cc.*		*γ per cc. 10*	
0	97	0	97	0	97	0	97
0.25	89	0.10	90	0.50	88	0.50	87
0.5	87	0.25	88	1.0	86	1.0	84
1.0	84	1.0	85	2.0	84	2.0	83
5.0	82	5.0	80	5.0	81	5.0	82
		10.0	80[‡]	10.0	81[‡]	10.0	82[‡]

[*] 10 cc. volumes incubated in Pyrex milk dilution bottles for 48 hours at 37°. The basal medium contains, per 10 cc., 2.5 cc. of amino acid solution,[2] 10 mg. of dextrose, 60 mg. of sodium acetate, 100 γ of guanine, adenine, and uracil, 1 γ of thiamine and riboflavin, 5 γ of nicotinic acid and nicotinamide, 20 γ of pyridoxine, 10 γ of calcium pantothenate, 0.01 γ of biotin and p-aminobenzoic acid, 0.1 γ of folic acid, 200 γ of inositol, 10 γ of Ca^{++} and Mg^{++}, 1 γ of Zn^{++}, Cu^{++}, Co^{++}, and Mn^{++}, 25 γ of Fe^{++}, 15.6 mg. of K_2HPO_4, and 1.4 mg. of KH_2PO_4. Initial pH, 7.8. Following sterilization of the medium, 1 γ of coenzyme I was added to each culture bottle. Putrescine was added as the dihydrochloride, spermidine and spermine as the phosphates. The additions are expressed in terms of the free bases.

[†] Per cent of incident light transmitted; uninoculated medium = 100.

[‡] Growth in presence of 10 γ of the indicated compound plus 1 mg. of orange juice solids.

degree of specificity for the tetramethylenediamine structure is thus indicated.

To our knowledge, this is the first demonstration of an essential nutritional function for one of the putrefactive amines, and indicates that putrescine and possibly additional compounds of this group play a much more important metabolic rôle than has been previously indicated.

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[2] Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).

AN INTERRELATIONSHIP OF PURINES AND VITAMIN B_{12}

Sirs:

Thymidine has recently been reported to replace vitamin B_{12} in stimulating growth of *Lactobacillus lactis* Dorner.[1,2] We have found that purines (or their derivatives) and thymidine are essential for growth of *Lactobacillus*

Effects of Purines and Derivatives on Growth Responses to Thymidine and Vitamin B_{12}

Test organism, *Lactobacillus lactis* Dorner,* incubated 28 hours at 37–38°.

Constant supplement	Galvanometer reading†					
	Guanylic acid, 100 γ per 10 cc.	Thymidine, 10 γ per 10 cc.	Guanine and hypoxanthine, 100 γ each per 10 cc.	Thymidine, 10 γ per 10 cc.	Adenine and guanine, 100 γ each per 10 cc.	None
Variable supplement, γ each per 10 cc.	Thymidine	Guanylic acid	Thymidine	Guanine and hypoxanthine	Liver concentrate	Liver concentrate
0	11	15	10	15	14	9
1	28		27		24	
2	41		36		33	29
3		21		24	41	31
5	62		50	37	51	42
10	65	40	57		59	52
20						59
30		53		48		
100		66		57		

* In a previously described medium (foot-note 1) from which purines were omitted unless otherwise noted.

† A measure of culture turbidity; distilled water reads 0, an opaque object 100.

lactis Dorner in the absence of vitamin B_{12}, as indicated in the table. The purine requirements are less specific than that of thymidine, which cannot be replaced by thymine. Of the compounds tested, guanylic acid was the most effective of single purines or derivatives; however, mixtures of adenine and guanine or of hypoxanthine and guanine were practically as active.

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[2] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 175, 475 (1948).

Three different concentrates of vitamin B_{12}[3] prepared by widely different processes replaced both purines (or derivatives) and thymidine in the nutrition of the organism; however, purines (or derivatives) had a slight sparing action which was never more than 2-fold. These results indicate that vitamin B_{12} is involved in the biosynthesis of purines (or derivatives) as well as thymidine but do not preclude the possibility that these substances in turn are also involved in the biosynthesis of vitamin B_{12}.

Dr. L. D. Wright has made available to us an organism, *Lactobacillus leichmannii* (ATCC 4797), which was used for assay during the isolation of the animal protein factor. In the nutrition of this organism, the animal protein factor can be replaced by thymidine.[4] In addition to this requirement, we have found that this organism requires folic acid for growth in a previously described medium containing purines.[1] The requirement for folic acid is replaced by thymine after a lag phase. Thymidine in the presence of folic acid adequately replaces the animal protein factor and also slowly replaces both folic acid and the factor after a lag phase. Hence, independent functions are indicated for folic acid and the animal protein factor, which presumably is identical at least functionally with vitamin B_{12}, in the biosynthesis of thymine and thymidine.

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[3] Rickes, E. L., *et al.*, *Science*, **107**, 396. Shorb, *Science*, **107**, 397 (1948).
[4] Wright, L. D., private communication.

HIGHLY VISCOUS SODIUM HYALURONATE*

Sirs:

Two important properties have distinguished sodium hyaluronate as isolated heretofore from that occurring in the natural state as in normal synovial fluid or in aqueous extracts of umbilical cord. It possessed only a fraction of the viscosity of the native fluids and gave no mucin clot with acidified serum. The proposed explanations of this difference were either (1) that the substance had become oxidatively degraded during isolation[1] or (2) that secondary valences had broken down during the process of isolation.[2] The first explanation did not appear reasonable, since the mucopolysaccharide isolated in an atmosphere of nitrogen still possessed a relatively low viscosity.[3] In the experience of this laboratory, the isolated polysaccharide was quite stable in air at pH 6 to 7 and in fact was always isolated by vigorous stirring in air for many hours. The existence of secondary valences in the native fluids, on the other hand, was not proved by experiment.

By extraction of homogenized human umbilical cords with 2 per cent phenol, clotting the mixture after dilution with dilute acetic acid, removal of protein, and avoidance of a pH greater than 7.5, we have isolated preparations of sodium hyaluronate which in 0.3 per cent solution in buffered sodium chloride at pH 6 have relative viscosities as high as 32 compared to those of older preparations of 3 to 4 in the same solvents. On addition of diluted serum and acidification, the new preparations give a typical mucin clot. Half viscosity was obtained with the new preparations as substrates of testicular hyaluronidase with one-tenth to one-twentieth of a turbidity reducing unit, while, with older preparations of the polysaccharide, 3 to 4 units of the enzyme were required to reach half viscosity.[4]

The highly viscous preparations of hyaluronate under standard conditions precipitate 15 to 25 per cent less serum protein *than older preparations*, as measured turbidimetrically. They show, furthermore, a deficit of ash of about 20 per cent as calculated either from the uronic acid or hexosamine values. Suspension of the dry powdered sodium hyaluronate in

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[2] Meyer, K., *Physiol. Rev.*, **27**, 335 (1947).
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[4] In contrast to a recently published method these preparations represent the total extracted polysaccharide of the cord and are not fractionated into viscous and non-viscous fractions (Hadidian, Z., and Pirie, N. W., *Biochem. J.*, **42**, 2 (1948)).

dry ammoniacal methanol for 3 weeks resulted in an increase in nitrogen of 0.52 per cent, (from 2.72 to 3.24 per cent), or about 20 per cent. Only half of this additional nitrogen was ammonia nitrogen, while the rest was firmly bound, presumably in amide linkage.

The experimental findings appear to be explained by the assumption of the presence of acid anhydrides which bridged the glucosidic polymer chains to giant molecules. These anhydride bridges, which occur to the extent of about 20 per cent of the total glucuronic acid molecules present, presumably are opened during the isolation by the older procedure.

It is possible that some of the biological reactions such as some spreading effects and the mucin clot prevention test[5] are due to the hydrolysis of these labile oxygen bonds. Whether similar linkages occur in other natural high molecular compounds of great lability, such as some proteins and desoxyribonucleic acids, remains to be seen.

We wish to thank Miss Hannah Weinshelbaum and Miss Anita Steinberg for their assistance.

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THE CONVERSION OF FORMATE AND GLYCINE TO SERINE AND GLYCOGEN IN THE INTACT RAT*

Sirs:

Glycine is generally agreed to be glycogenic. This net deposition of glycogen could be accounted for by the pathway via serine to pyruvate shown in Scheme I.

$$CH_2NH_2COOH + HCOOH \rightarrow CH_2OHCHNH_2COOH$$
$$\rightarrow CH_3COCOOH \rightarrow glycogen \qquad (I)$$
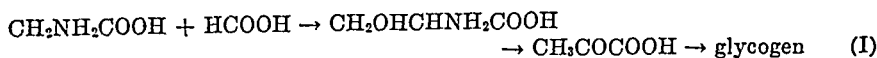
Evidence has been reported for the *in vitro* conversion of glycine to serine[1] and serine to pyruvate.[2] However, in the intact animal, the evidence was to the contrary.[3]

We have investigated this mechanism by degrading liver serine and glycogen isolated after the simultaneous administration of glycine labeled with C^{13} in the carboxyl group and formate containing C^{14}. According to Scheme I, the serine carboxyl group is derived from the corresponding group of glycine and should contain excess C^{13}, while the β-carbon atom, which is formed from formate, should be labeled with C^{14}. The glycogen should contain C^{13} in the 3,4 positions and C^{14} in all carbons, with the highest concentration in the 1,6 and lowest in the 3,4 positions. C^{14} in the 3,4 positions would result from fixation of $C^{14}O_2$ formed from the formate, while the reversible transformation of pyruvate into a symmetrical 4-carbon dicarboxylic acid would introduce the C^{14} of the β-carbon into the α position of pyruvate and the 2,5 positions of the glycogen.

Four fasted rats weighing a total of 571 gm. were given 5 mM of glycine by stomach tube and 0.125 mM of formate intraperitoneally per 100 gm. The latter dose was repeated at the end of the 5th hour. After 14 hours the animals were sacrificed. Glycogen was extracted from the livers with trichloroacetic acid and degraded by the procedure of Wood *et al.*[4] Serine was isolated from the neutral amino acid fraction of the liver hydrolysate as the *p*-hydroxyazobenzene sulfonate, m.p. 208–211° (decomposition) and degraded (Scheme II).
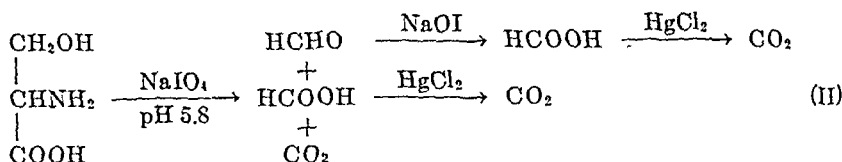
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[4] Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945)

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & & \text{HCHO} \xrightarrow{\text{NaOI}} \text{HCOOH} \xrightarrow{\text{HgCl}_2} \text{CO}_2 \\
| & & + \\
\text{CHNH}_2 & \xrightarrow[\text{pH 5.8}]{\text{NaIO}_4} & \text{HCOOH} \xrightarrow{\text{HgCl}_2} \text{CO}_2 \qquad\qquad \text{(II)} \\
| & & + \\
\text{COOH} & & \text{CO}_2
\end{array}
$$

The results of the carbon analyses, shown in the table, are in accord with Scheme I. Serine and glycogen contained considerable C^{13} and C^{14}. In serine, all of the C^{13} was located in the carboxyl carbon (Column 2), while

Compound	Total C (1)		COOH or 3,4* (2)		α or 2,5* (3)		β or 1,6* (4)	
	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡	C^{13}†	C^{14}‡
Serine..............		133	0.78	4	0.00	20	0.00	377
Glycogen................	0.27	343	0.81	212	0.00	321	0.00	512

The original formate and glycine carboxyl carbon contained 1.35×10^5 counts per minute per mg. of C and 9.98 atom per cent excess C^{13} respectively. 48 per cent of the formate C^{14} was recovered in the respiratory CO_2.

* Positions of the glucose unit of the glycogen.

† Atom per cent excess.

‡ Counts per minute per mg. of C.

most of the C^{14} was in the β position (Column 4). In glycogen the C^{13} was restricted to the 3,4 carbons (Column 2), while the C^{14} was distributed throughout the molecule with the highest concentration in the 1,6 positions. The absence of C^{13} from the β position of serine proves that this carbon does not arise from formate via CO_2 fixation.

This experiment demonstrates the likelihood of a pathway for the conversion of glycine and formate to glycogen via serine and pyruvate.

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PANCREATIC CARBOXYPEPTIDASE; A METAL PROTEIN*

irs:

It has been demonstrated that many exopeptidases are metal proteins as ldged by the activation produced by specific metals, by inhibition studies ith metal poisons, and by the slow reactivation by metal ions of the par- ally purified proteins (time reactions).[1]   The crystalline carboxy- eptidase[2] of bovine pancreas has appeared to be an exception to this, but , has not yet been studied from this viewpoint.   We now wish to report iat 5 times crystallized carboxypeptidase is inhibited by typical metal

Effect of Metal Inhibitors on Carboxypeptidase

The tests were performed at 25° with carbobenzoxyglycyl-L-phenylalanine (0.05) as the substrate in the presence of 0.067 M phosphate buffer at pH 7.4.   Neutral- :ed solutions of the inhibitors were mixed with the enzyme, allowed to stand for 5 iinutes, and then added to the buffered substrate solution.   Each test solution con- iained about 0.7 γ of protein N per cc. K is the first order velocity constant.

Inhibitor	Time	Hydrolysis	$K \times 10^3$	Inhibition
	hrs.	*per cent*		*per cent*
Jone	0.5	33	5.8	
	1.0	53	5.5	
	1.75	70	5.1	
).008 M sulfide	26	1		100
).008 " cyanide	1.0	22	1.8	80
	2.0	25	1.0	
	5.5	40	0.7	
).008 " azide	1.0	52	5.2	0
	1.75	71	5.1	

poisons.   It is apparent from the table that the enzymatic action is com- pletely blocked by 0.008 M sodium sulfide, and is strongly and progressively inhibited (approximately 80 per cent) by 0.008 M sodium cyanide.   Sodium azide (0.008 M) has little or no effect on the enzyme.

The inhibition of this enzyme by these typical metal poisons suggests that carboxypeptidase is a metal protein like other exopeptidases.   How- ever, while most of the exopeptidases are exceedingly labile and lose their metal on prolonged dialysis or after mild purification procedures, the metal

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[2] Anson, M. L., *J. Gen. Physiol.*, **20**, 663 (1937).

in carboxypeptidase must be much more firmly bound to the protein. Examination of the residual dry ash of our enzyme preparation in the arc of a sensitive spectrograph[3] has shown the presence of significant amounts of magnesium, and traces of iron and copper. Zinc, manganese, and cobalt, elements which are concerned in the activity of other peptidases, could not be detected. Moreover, no barium or lithium, which is used in the preparation and recrystallization[4] of the enzyme, could be found. The present evidence points towards magnesium as being the metal concerned in carboxypeptidase activity.

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